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August 1976

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AUGUST 1976

PREPARED FOR

OFFICE OF TOXIC SUBSTANCES
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The English language literature for the period 1954 to May 1974 has been searched. A computer title search, contacts with scientists currently engaged in related research, and bibliographic references contained in individual papers were pursued. Copies of articles were obtained and reviewed under the following groupings: 1) Use of Fertilized Eggs in Studies on Chemicals, 2) Use of Isolated Organs and Tissue in Studies on Chemicals, 3) Use of Mammalian and Avian Cell Culture in Studies on Chemicals, 4) Use of Bacteria, Fungi, Protozoa, and Plant Cells in Studies on Chemicals. An attempt has been made to include all systems within these headings. Where such information was available, the applicability of those in vitro test systems has been evaluated.

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REFERENCES

ABSTRACT

The English language literature for the period 1954 to May 1974 has been searched by several methods including a hand search, a computer title search, contacts with scientists currently engaged in related research, and bibliographic references contained in individual papers for articles in which *in vitro* methods were used to detect, define, and/or quantitate biological effects of chemicals. Copies of these articles were obtained and reviewed under the following groupings:

(1) Use of Fertilized Eggs in Studies on Chemicals

(2) Use of Isolated Organs and Tissue in Studies on Chemicals

(3) Use of Mammalian and Avian Cell Culture in Studies on Chemicals

(4) Use of Bacteria, Fungi, Protozoa, and Plant Cells in Studies on Chemicals

An attempt has been made to include all systems that have been used within these headings to assess the biological activity of chemicals. Where such information was available, the applicability of those *in vitro* test systems has been evaluated.

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I

CONCLUSIONS

In vitro methods have been reported for the assessment of nearly every known manifestation of toxicity of a chemical. The endpoint in the in vitro system, however, is rarely descriptive of the toxic signs noted in the whole animal. Thus, in vitro systems are valuable tools for screening large numbers of chemicals for a single toxic manifestation, e.g., a carcinogen. On the other hand, to define the toxic properties of a single chemical substance would require so many in vitro systems as to be impractical.

In vitro test systems have proven very valuable tools in the study of mechanisms of action, in drug metabolism, and in defining nutritional and hormonal requirements.

In vitro systems have served as useful analytical methods for the control of potency of antibiotics and for detection of antibiotic residues or antibiotic contamination.

In the field of toxicology, limited uses of in vitro methods have been employed in areas such as evaluation of plasticizers and other components of medical devices fabricated from polymeric materials. The use of fertilized eggs has enjoyed some attention in studies of teratogenic potential of chemicals, but this method is still regarded by toxicologists as of marginal usefulness. The many methods that are being proposed for evaluation of both mutagenic and carcinogenic potential have yet to be shown to be sufficiently predictive to be recommended as routine screening procedures.

A serious problem arises in the use of in vitro systems to arrive at any reliable estimate of a quantitative expression of toxicity. Concentrations shown to be effective in in vitro systems in producing a measurable endpoint are often two or three orders of magnitude higher than can be shown to be effective in whole animal systems. A cytotoxic effect of a chemical at these exaggerated concentrations compared with real life is viewed by the toxicologist with considerable reservation.

In vitro systems results, for the most part, reflect the acute or single dose effects of a given chemical. Aside from the possible utilization of in vitro systems for mutagenic and carcinogenic screening, such systems have not been shown to be very useful in predicting the results of the long-term administration of a chemical. The usefulness of such systems in predicting such toxic manifestations as eczematous sensitization, hypertension, chronic kidney disease, cardiovascular disease, blood lipid deposition in the vascular system, cataracts and other eye defects, eighth cranial nerve damage, reproductive disorders, and cirrhosis of the liver is yet to be demonstrated.

II

INTRODUCTION

A number of events in the past several years has alerted first the scientific community and subsequently concerned citizens' groups to possible hazards associated with the widespread distribution of synthetic and industrial chemicals in the environment. Although Rachel Carson's book "Silent Spring" (1) contains a number of excessive and exaggerated statements, it did serve to alert the general public to the potential hazards that could arise from the continued heavy and unrestricted use of DDT. Since the publication of this book, accounts of other chemicals that may be producing long-term subtle effects have appeared in the news media and include such chemicals as the polychlorinated biphenyls, the dioxins, the halogenated insecticides such as aldrin, dieldrin, mirex, and hexachlorobenzene, arsenic, cadmium, mercury, and most recently a widely used industrial chemical, vinyl chloride. Less publicized have been adverse findings with bischloromethyl ether, benzidine, and ethylene dibromide.

The above-mentioned chemicals are not newly synthesized chemicals. They have been around for years and some biological data on each of them were available long before the chemical became notorious. How then did the "toxicological surprise" arise? The answer in each case is that the growth in the use of the chemical far surpassed the growth in the understanding of potential toxicological and environmental hazards. Increased use levels place many more populations at risk. In a few instances, use of the chemical has become so worldwide that all existing animal and plant life are at risk. Thus a very sensitive organism may disappear forever.

Why does the understanding of the toxicology of a compounds lag so far behind its expanded use? Among the answers to this question are:

- Costs to conduct valid long-term experiments in animals are prohibitive.
- Time required to conduct such experiments is excessive.
- The sensitivity of the animal model test system is poor.
- In a test system as complicated as a whole animal, a single non-lethal effect may be obscured by massive amounts of extraneous normal biological variation.
- Unique expressions of toxicity continue to appear that previously developed protocols failed to detect.

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An alternative approach to the study of the toxic properties of a chemical that has occurred to many investigators is to make use of in vitro test systems. Thus, conventional animal model test systems could be eliminated in whole or in part by the substitution of, or supplementation by, in vitro test systems. The desired goals of reduction in costs, more rapid evaluation, improved sensitivity, increased test population numbers, and diversification in endpoints are believed to be achievable using in vitro systems.

The purpose of this survey is to determine, based upon a review of the published literature and of ongoing research efforts, the strengths and weaknesses of in vitro systems as test methods for the assessment of toxicological hazards.

For purposes of this review, in vitro test systems have been confined to the following:

- Fertilized Eggs
- Isolated Organs and Tissues
- Mammalian and Avian Cell Culture
- Bacteria, Fungi, Protozoa, Plant Cells

Historically, investigators have been attracted to in vitro test systems because of one or more of the following considerations:

- Apparent simplicity of an in vitro system.
- The possibility of using cells or tissues from the species potentially at risk, thus avoiding the uncertainties in extrapolating from one species or strain to another.
- The possibility of conducting experiments on vast numbers of organisms as opposed to small numbers of whole animals.
- The possibility of taking advantage of rapid cell turnover rates so that many successive generations can be observed over a relatively short period of time.
- The ease of investigating a large number of biochemical reactions taking place in pure cell cultures or in isolated organs or tissues composed of relatively few cell types.

However, the characteristics of in vitro systems that have attracted investigators have also been responsible for the limited value that toxicologists have placed upon the findings in such systems. In more recent years, the investigators using in vitro systems are finding that their apparent simplicity is deceptive. It is now realized that the cell at risk as a result of exposure to a chemical is often a cell which is affected by a metabolite of the chemical or by secretions of other cells in response to chemical exposure. The in vitro cell system under study may not be affected in any measurable way by the parent chemical. It is also being realized that in the intact animal, the concentration of a chemical at a particular site is determined by the interaction of a large number of dynamic processes, along with the physiochemical properties of the compound. These include the following:

- Rate of absorption from the site of entry.
- Protein binding, binding to formed elements in the blood.
- Locus and rate of metabolic conversion.
- Routes and rates of excretion from the body of both the parent compound and its metabolites.
- Oil/water partition coefficient.
- pKa value for parent compound and its metabolites.
- Membrane diffusion characteristics.
- Site of tissue specific binding.

In any given in vitro test system, only one or two of the above factors are operative so that effects that may be observed in vitro may not reflect accurately the effects that will be observed in the intact animal. On the other hand, the absence of these interacting factors can be taken advantage of in the development of in vitro bioassay systems for the comparison of large numbers of closely related chemicals.

In any review of in vitro test systems, it should be remembered that the classical work of Otto Loewi beginning in 1921 (2) demonstrating that acetylcholine is a neurotransmitter was carried out using isolated perfused frog hearts. The production of abnormalities in the developing embryo as a result of the administration of thallium was first demonstrated by Karnofsky in 1950 using the fertile chicken egg (3).

There is an urgent current need for valid, reliable, and simple in vitro methods that will be predictive not only of teratological, mutagenic, and carcinogenic effects but also of irreversible organ damage such as emphysema, cardiovascular disease, nephritis, and myelin degeneration. Chronic collagen disease and the auto-immune manifestations should be subject to early detection also. Effects of chemicals on mineral metabolism, endocrine secretion, and cellular uptake of essential substrates have received some study in in vitro systems (Section IV). Increased efforts in this area should improve materially our understanding of the potential environmental hazards of widely dispensed chemical, particularly on the reproductive processes.

Three recent communications (4, 5, 6) provide some insight on the complex considerations involved in the design, conduct, and most importantly the interpretation of the results of in vitro systems which attempt to predict carcinogenicity. The following quotes seem of particular importance:

1. ". . . as chairman of the AAAS Committee on Scientific Freedom and Responsibility, John Edsall is in a position to influence public policy . . . He notes in his letter of 18 July 1975 the finding that some carcinogens are mutagenic in bacteria. This has been interpreted to mean that those carcinogens cause cancer by somatic mutation and has been taken by many as support for the venerable hypothesis that the malignant transformation of cells is a mutational event. In addition, the screening of compounds for their capacity to cause bacterial mutations has been adopted by a number of laboratories as a means of indicating carcinogenic potential. The implied relation between mutagenesis and carcinogenesis still needs careful scrutiny with regard to its scientific validity and also because of its implications for public policy . . .

   "Acceptance of screening for carcinogenicity by determining mutagenicity lends tacit support to the hypothesis that malignant transformation of cells is caused by somatic mutation. This hypothesis has been tested explicitly in several experiments and has been found wanting in each case." (4)

2. "On the basis of our work, and the lines of evidence noted below, I find compelling the theory that chemical carcinogens cause cancer through damage to DNA (somatic mutation).

   1) It is known that cell regulation can be easily altered by mutation and that a heritable change in cell regulation is a characteristic property of a cancer cell.

   2) The theory is simple and accounts for what is known about the molecular biology of cancer and chemical and radiation carcinogenesis.

   3) Studies on the genetics of cancer suggest this idea.

   4) Human mutants exist who are extremely prone to cancer and who lack DNA repair systems.

5) There is a correlation between capacity for repair of DNA damage and the occurrence of organ specific cancer.

6) Active forms of many carcinogens are electrophiles capable of interacting with DNA.

7) Ninety percent of carcinogens are mutagens; it is our hypothesis that the aromatic part of aromatic carcinogens is involved with stacking interactions in DNA, causing frameshift mutations.

8) The well-known human carcinogen asbestos has recently been shown to be an efficient breaker of chromosomes . . .

"It is likely that mutation (imitation) is not the only cause of cancer and that a few environmental chemicals may well work through other mechanisms; however, there is not much evidence that such environmental chemicals (or even viruses) are contributing in a major way to human cancer. Rather, the evidence indicates that chemicals are radiations in the environment (cigarette smoke, ultraviolet light, nitrosamines, and so forth) damage DNA and that this damage, incurred throughout our lifetimes, is the initiator of most of human cancer. DNA damage is quite likely to be a major contributor to birth defects, aging, and heart disease as well." (5)

3. ". . . The most subtle of these genetic changes, and yet the one likely to have the most far-reaching consequences for man, is point mutation, an alteration in one of the nucleotide bases of the DNA . . .

"In vivo testing in mammals probably has the greatest relevance to the human situation, but tests such as the specific locus test in the mouse require enormous numbers of animals and are consequently prohibitively expensive and time-consuming for the routine testing of drugs and chemicals. In addition they are less sensitive than microbial tests. Other mammalian tests, including the dominant lethal assay, and tests using Drosophila detect gross chromosomal damage rather than point mutations.

"Bacterial tests offer many advantages over mammalian systems in that they are far cheaper, very rapid (the results are obtained usually within two days) and extremely sensitive. Indeed microbial tests probably represent the only system suitable for screening large numbers of chemicals for mutagenic activity. It has been suggested that the wide phylogenetic difference between bacterial and mammals precludes the use of the former in mutagen testing, but this objection may be countered by the fact that the target molecule of mutagens is DNA, which is substantially the same whether the cell is eukaryotic or prokaryotic.

"Most of the bacterial tests for detecting mutagens utilize bacteria bearing a mutation in a gene involved in the synthesis of a particular amino acid; hence the bacteria (called auxotrophs) require an external supply of the amino acid for growth. Spontaneously, and at low frequency, some of the bacteria revert, or back-mutate, to the wild-type, able to grow on media lacking the amino acid. Mutagens can induce such back mutations and so an increase in the number of revertants over the number of spontaneous back mutations can be used as an index of the mutagenicity of the compound under test. The best known of these back-mutation systems are the histidine-requiring mutants of _Salmonella tiphimurium_ developed by Ames and the tryptophan mutants of _Escherichia coli_ described by Bridges. In both systems the sensitivity of the test has been increased by utilizing derivatives of the tryptophan or histidine auxotrophs deficient in one or more of the mechanisms by which the bacterial cells repair damage to DNA. The most useful of these are _uvr_ strains.

"As it stands, this test has several disadvantages, not the least of which is the considerable difference in permeability between bacterial and the mammalian cells. In the bacterial test, a negative result may be due not to a lack of mutagenic activity on the part of a chemical but to its inability to penetrate the cell wall and membrane and reach the DNA within the bacterium. To combat this, Ames _et al_. obtained deep rough derivatives of _S. typhimurium_ strains, the cell walls of which were deficient in lipopolysaccharide components and were thus more permeable to various mutagens, including dibenz(a,h)anthracene.

"Perhaps, the most important drawback of the bacterial test described above is that it does not take into account the major differences in metabolism between mammalian cells and bacteria (e.g., the cytochrome P-450 drug-metabolizing system of mammalian liver, a system which has no counterpart in the bacterial cell). Thus a non-mutagenic chemical metabolized by such enzymes to an active mutagenic species will not be detected by the simple bacterial system. (Obviously the converse is also true --a mutagen inactivated by mammalian enzymes will give a 'false positive' when tested with bacteria.) Thus mutagens such as acetylaminofluorene and dimethylnitrosamine, which are not mutagenic _per se_ but require activation by liver enzymes, do not revert the histidine or tryptophan auxotrophs. This problem has been partially overcome by two methods, the host-mediated assay and the activation of mutagens by liver homogenates . . .

"The second approach to the problem of mutagen activation, the use of liver homogenates or microsomal fractions, looks more promising. This group (Ames group) showed that several compounds that do not normally mutate S. typhimurium in vitro, will do so when a rat-liver homogenate is incorporated into the agar plate. Unfortunately, this investigation was confined to a very restricted range of mutagens, mostly polycyclic aromatic hydrocarbons, so it is not certain how well the system responds to other types of mutagen, although dimethyl- and diethylnitrosamines are known to be genetically active by this method. One of the major problems with this method is the choice of animal for use as the source of the liver extract. Malling & Frantz have shown that for dimethylnitrosamine there is a remarkable difference in metabolic activity not only between liver homogenates derived from the rat and mouse but also between those from different strains of mouse. Furthermore, the method does not take into account compounds activated by enzymes other than those associated with the liver. To be certain of detecting such mutagens, homogenates of other organs, such as the lung, intestinal epithelium and kidney, would also have to be used . . .

". . . to ensure that all environmental mutagens are detected, we may have to face the daunting task of using many different strains of bacteria in combination with liver homogenates from a variety of animals -- besides the possible use of homogenates of other organs . . .

"In spite of the limitations discussed in this article, microbial mutagenicity tests are obviously the only means available for screening large numbers of chemicals and drugs for mutagenic activity, since in terms of speed and sensitivity they are vastly superior to in vivo tests in mammals . . .

"Obviously, for a full definition of the mutagenic potential of a chemical it is necessary to determine many more facts, such as the mutagen concentration at the site of action, the active metabolic products and their rate of absorption, metabolism and elimination." (6)

III

METHODS EMPLOYED FOR SURVEY

In general, the survey covers the English language literature from 1954 through May 1974. A few key publications have also been included that have appeared since that time.

The literature review was accomplished through a hand search of more than 20 of the most widely used journals in toxicology, pharmacology, and cell biology; a computer title search; contacts with those having ongoing research listed with the Smithsonian; and attendance of a number of scientific meetings during 1973-1974 at which in vitro methodology was discussed. Additional references were reviewed based upon bibliographic citations in individual papers. Finally, the outside experts that were involved either in the early direction of the review or in the review of the draft monographs provided a few references that had escaped the attention of the survey team.

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TEMPERATURE AND HUMIDITY

Environmental variables need to be standardized. Temperatures of incubation are stated in papers as °F or °C and range from 35°C (17) to 38°C (18). In most instances incubation temperatures range from 37°C to 38°C. Abbott and Craig (19) have emphasized optimal conditions required for incubation of chick eggs for maximum hatchability. They state that differences in temperatures are required in still-air (102-103°F) versus forced draft incubators (99.8°F).

The rate of development may be dependent on environmental temperatures.

Humidity is a variable which is seldom recorded. Reported values range from 65% (20) to 86-90%.

TURNING OF EGGS

Another condition of incubation not generally explicitly stated in methods section of papers is turning eggs several times daily. Abbott and Craig state that eggs must be separately hand turned or the rack may allow for turning entire trays at once. Among the few studies listing this parameter specifically are Walker (7), Pagnini, et al. (21), and Mawhinney, et al. (22). This is important for the proper hatching of the chick. However, shaking or jarring eggs before incubation is stated to produce 60% mortality between the second and third days (Hamilton, 23), This is said to result from a failure of the vitelline vessels to organize out of the blood islands.

TIME AND ROUTE OF ADMINISTRATION

Time and route of administration of test materials are important variables. Time of administration ranges from pre-incubation injection (8, 9, 14, 16, 24, 25, 26, and 27; McLaughlin, et al. 1963 (28) to injection at mid-incubation (15 days). Injection before incubation has been stated to yield excess mortality. Khera and Lyon (16) had an average survival of 48% during the first four days of incubation after pre-incubation injection, as compared with 75-95% survival of eggs injected after four or more days of incubation via the yolk sac using propylene glycol as a solvent. The high mortality noted by Khera and Lyon at pre-incubation injection contrasts with the low mortality observed by Verrett (personal communication). The high mortality at pre-incubation injection reported by some may be a result of too vigorous twisting or turning of the egg with a disturbance of the blood islands as described in the preceding section. Khera and Lyon (16) attribute this to differences between strains of eggs.

CANDLING PROCEDURES

Candling (transillumination) of eggs at regular intervals is done to evaluate the viability of the embryo. Intervals practiced vary as follows:

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- Daily (7, 14, 29)
- Fifth day and daily thereafter (8, 9, McLaughlin)
- Regularly, interval not stated (20, 30, 31)
- On days 4 and 6 (24)
- Day after injection, days 5-6, and every 1-4 days (16)
- On days 7, 14, 18 (32)
- On day 5 and 10 (33)

Several investigators prepared "Scotch Tape" (12, 34, 35) or coverslip windows (36, 37) for direct observation of the embryo at injection and afterward.

STAGE OF DEVELOPMENT

Injections given at the pre-incubation stage may interfere with the initial processes of embryogenesis. Studies which use eggs pre-incubated for various periods of time therefore avoid chemical effects on initial stages of embryonic development. It is very common to inject at 3-4 days of development (30, 24, 16, 32, 20, 21, 38, 39, 33, 40, 41, 11, and 22).

The state of the embryonic development of the chick at the time of injection may be described by reference to the 44 stages as listed by Hamberger and Hamilton (42). Reference to number of somites (43) or a feature of development (44) at the time of injection is infrequent. At four days the vascular system and internal organs have formed in the chick; however, formation of bone has not yet begun. Embryogenesis of the chick is much more rapid than of the rat at corresponding gestation age, although the gestation and incubation periods are comparable. Thus the closing of the neural fold in the chick occurs at one day, as compared to 10 days in the rat. Liver and pancreas appear in three days as compared with 12 days in the rat.

Many studies have used the 8-15 day incubation period (25, 45, 20, 46, 14, 29, 47, 17, 15, 40, 11, and 48), in most instances as part of a series (16, 25, 20, 14, 17, 49, 40, 11, and 12) to compare toxicity.

Other pre-incubation time intervals prior to injection that have been used in studies include 24-48 hours (24, 20, 34, 36, 33, and 50); 49-72 hours (7 and 35), and 5-7 days (51 and 22).

SITE OF INJECTION

The site of injection is a variable that has received some attention. In eggs that have not been pre-incubated, only the yolk sac, air cell or albumin are available. The most frequent site of injection is the yolk sac (30, 24, 31, 16, 25, 26, 32, 51, 21, 20, 29, 46, 52, 39, 33, 40, and 41). This method has been subject to the criticism that material administered is unevenly distributed in the yolk because of solubility problems. According to Walker (7) injected material may rise toward the embryonic disc or never expose the embryo according to the specific gravity of the material. Walker (7) describes the technique and use of a yolk replacement method to insure uniform distribution of material. He found a lower mortality rate with high doses of pesticides than by the conventional yolk sac injection method, but higher mortality rate with low dose levels. There was also a higher rate of malformations with the yolk replacement method. Verrett (personal communication, 1974) states that the technique of Walker is not representative of the art. In her experience the technique results in almost 100% of abnormal birds even with controls. A technique was described by Klein _et_ _al_. (39) for perfusion of four-day embryos with dilute yolk solutions in artificial medium containing salts, vitamins, amino acids and 8% yolk. This permitted survival until 96 hours when an experiment was terminated. No foreign chemicals were studied in this system.

The air sac is a route favored by some experimenters (14, 8, 9, 41, 53, 22, 54, and 55). In a comparative study, Verrett _et_ _al_. (54) found that doses of aflatoxin showed a greater toxicity (mortality) when injected into the air cell than by yolk injection. Material deposited in the air sac gains access to the embryo by the egg membrane and albumin.

The chorioallantoic membrane develops during the second 24 hours of incubation when the head fold appears. This membrane has been utilized by many for studies of embryonated eggs pre-incubated for 4-8 days. Ridgeway and Karnovsky (56) had shown that heavy metals are, in general, more toxic when administered into chorioallantoic membrane than by yolk sac. Others have also utilized this route of administration (12, 15, 17, 35, 37, 38, 47, 49, 57, and 58).

Extra-ovo (tissue culture) methods have been utilized for the study of potentially toxic materials at special target sites. This is generally done after the tissue or organ has differentiated from its anlage. Hay (59) has reviewed the dependence of each stage of differentiation of a given tissue or organ upon a preceding stage and type of tissue by means of induction. This problem has also been attacked by means of tissue culture techniques sometimes with several types of tissue together in the culture flask (59). Chemicals (drugs) and tissues used for such studies include LSD, whole mount at one day (43), cortison, growth of femur of seven-day embryo (13), Vit D_3, intestine, 20 days (60); insulin, tibia, 10 days (61), antimetabolites whole mount connective tissue (lathyrism), eight days (62); actinomycin D, early axial development, 11-13 somite stage (63), propranolol, pre-neural heart, 50-55 hours, 80-85 hours (64); and various drugs on heart primordia (65).

END POINTS

Mortality

Most studies have evaluated toxicity by mortality noted, or have recorded this as ancillary data. Studies in which mortality was compared at several dosage levels or with an attempt made to calculate the LD_{50} have been performed on phosphate and carbamate pesticides (7), a variety of pesticides (16), solanine (32), alkylating agents (20), vaccine (29), dinitrophenol (14), benzimidazoles (8, 9), azaserine (36), chlorpromazine (33), beta-aminopropionitrile (58), volatile industrial chemicals (66), and a variety of chemicals (28).

Growth and Development

The incidence of teratological effects and effects on gross development was the major emphasis of a number of studies (7, 31, 32, 20, 43, 34, 14, 38, 52, 9, 36, 35, 37, 33, 40, 41, 11, 62, 67, 68, 69, 70, 71, 58, 55, 66, and 28).

The effect of test compounds on the development of a special target site was emphasized in some studies. Such studies included the effects of LSD on development of neural tube (43), dinitrophenol on lens (14), beta-aminopropionitrile on connective tissue (38) and on skeleton (58), semicarbazide and insulin on skeleton (58, 69, 70) and cardiovascular drugs on heart (65).

Tritiated thymidine uptake has been used as a general measure of growth and development (72 and 22).

Physiological Activity

The effect of test compounds on physiological activity was also tested in a number of studies: heart - heart rate (64); bones - growth rate (13), collagen content (49, 61), histochemistry (58); hematopoetic system - tritiated thymidine uptake (30), ALA-synthetase induction (53); immune system - gamma globulin effect (47), antibody production (15); intesting - induction of calcium binding protein (60), and motor junction as a measure of muscle strength (25).

CORRELATION OF IN VITRO WITH IN VIVO METHODS

In general fertile chicken eggs may have a similar or enhanced sensitivity to chemicals as compared to rats depending on technique employed and chemical under study (24, 73). McLaughlin *et al.* (66) state that the toxicity of the industrial solvents as determined with their method correlates with threshold limit values, guides used in industrial hygiene practice. Verrett (personal communication) states that she has compiled a list of 30 drugs in which mammalian and chick teratology were studied and in which there was teratological information in the human. She states that there is only one case where the chick and mammals did not show a teratologic response when it was found in the human. In all cases chick and mammal showed responses which, however, were sometimes different.

Recently the chick embryo was adapted for the purpose of bioassay of aflatoxin by the Association of Official Analytical Chemists because of its great sensitivity to the toxin.

The chick embryo and other fertilized egg systems have a special utility in evaluating possible effects of chemicals as environmental pollutants. For example, there have been several reports regarding agricultural chemicals which inhibit hatchability of bird's eggs (74). The use of an appropriate technique to evaluate this potential hazard thus assumes economic significance.

One of the significant factors to be considered in correlating environmental effects with experimental data is the deposition of a toxic chemical in the egg. In nature, contamination of the egg would occur via the oviduct of the hen. The yolk and albumin then serve as reservoirs for the toxic agent. If deposited in the egg via the oviduct, trace amounts of a toxic chemical would be uniformly distributed. Chemicals intentionally injected into the yolk sac may not distribute uniformly or be available at early stages of embryogenesis to produce a toxic effect. Guthrie and Donaldson (26) have shown that 40% of the total radioactivity of the one-day old chick which develops from an egg injected with radiolabeled DDT by the technique of McLaughlin (28) is found in the yolk sac at hatching. The yolk sac is incorporated in the body of the chick before hatching and provides a source of nutrition. Thus the toxic effect of a chemical injected in the yolk sac may only result after absorption at the time of hatching. It would appear that injection at 4-10 days into the chorioallantoic membrane is a preferable technique for conducting toxicity tests. It has been shown that a low incidence of mortality occurs in saline injected controls at this age as compared with pre-incubation treatment. Moreover, the toxic material gains immediate access to embryonic tissues.

The concentration of dieldrin has been measured in blood of embryos and chicks hatching from eggs treated with this compound (Koeman *et al*., 1965). Dieldrin was injected by the method of McLaughlin *et al*. He also found that the concentration of dieldrin in the blood of the 0-6 hour old chick was little greater than that in the 14 day old embryo, which indicated that the absorption process of the yolk sac did not cause an important increase in the concentration of dieldrin in the blood. They state that a sufficient period after hatching is required to determine whether the chick is susceptible to the test material.

Invertebrate Eggs

Toxicity tests have been conducted using invertebrate eggs as test subjects. Two papers are of particular interest in this respect:

1. Various types of carcinogens were tested using the inhibitory effect of hatching of *Artemia Saline* (brine shrimp)

as the criterion for an effect (Buu Hoi and Pham-huu Chang, 75). Fourteen compounds belonging to seven different chemical groups were selected. Thirteen of these compounds were confirmed as carcinogens in animal experiments; the other compound was structurally related to a carcinogen. In general, the inhibitory or stimulatory effect on hatching did not correlate with the carcinogenic effect of a test compound. Within the epoxide series, however, a clear cut difference was found which correlated with skin tumors produced by painting on mice.

2. The fertilized sea urchin egg was the test object for a study on the influence of fractions of a tobacco smoke condensate on early development (76). The stage of development attained by treated eggs was the criterion for a toxic effect. The eggs were sensitive to fractions containing unsaturated hydrocarbons and other substances. They were not, however, sensitive to nicotine or other polycyclic hydrocarbons still present in these fractions. The sea urchin egg was therefore considered to be useful for subfractionation studies in the isolation of toxic materials found in tobacco smoke condensate.

Other papers which have appeared concerned special classes of chemicals and special effects on the eggs and/or larvae of lower organisms. Thus the sea urchin was the subject of study of inhibitors of oxidative phosphorylation (77) which induce birefringence of the mitotic apparatus, inhibition of glucose metabolism (78), inhibition of synthesis of RNA and gene expression by actinomycin (79), inhibition of cleavage by puromycin and inhibitors of cyclic AMP (80) and bizarre development termed an animalizing effect after exposure to Evans Blue (81). Insect larvae (attagenus piceus) were test objects for inhibitors of cholesterol synthesis (82). In this work test compounds inhibited growth (weight gain) of the larvae. Zebra fish embryos (Brachydanio, rerio, Hamilton) were test objects for a large number of steroidal estrogens (83). Compounds were evaluated by an ED_{50} determination based on the cytostatic effect. No relationship was found between estrogenic and cytostatic potency.

In experienced hands, the use of fertilized chicken eggs for teratological screening provides useful information. The record would indicate that the technique has limited usefulness in screening for other types of toxic effects.

The use of the sea urchin egg in working out a mechanism of action of a toxicant is at the present the best place for this technique.

B. USE OF ISOLATED ORGANS AND TISSUE IN STUDIES ON CHEMICALS

INTRODUCTION

In order to bridge the gap between the effects of a chemical on the cell and the effect on the whole animal, isolated organs or organized tissues are used. The chemical is introduced by one of three methods, perfusion through the vascular system, adding it to the bathing fluid in which the tissue is suspended, and adding the material to tissue slices in an apparatus such as the Warburg. Among the parameters commonly measured as indices of organ function are rhythmic contractions, fluid transport and flow, organ secretion, oxygen uptake, carbon dioxide production, and incorporation of radiolabeled substrates.

LIVER

The liver is a large glandular organ which converts most sugars into glycogen and is concerned with storage of fat and excretion of chemicals. For the purposes of this survey on isolated organs, liver homogenates and enzyme induction studies were not considered pertinent. Because of the role of the liver in detoxification and metabolic fate of materials, the isolated perfused liver and liver slices are important in in vivo test systems.

The isolated perfused liver is useful for studying the metabolism of chlorinated hydrocarbons (84). Cole et al. (49) used radiolabeled endrin and dieldrin in the perfusate and compared the fat storage of these two compounds in the intact rat and the isolated perfused rat liver. A modified method of Miller (85) was used. The radiolabeled insecticide dissolved in 0.1 ml acetone was added to the circulating perfusate to provide an initial concentration of 0.003 mg/ml or 0.0003 mg/ml insecticide in the perfusate; 0.003 mg/ml approximated the initial in vivo concentration in the blood. The results obtained in the perfusion experiments were very similar to those in vivo, indicating that the liver was the major controlling factor in different rates of excretion of radioactivity from endrin 14_C and dieldrin ^{14}C.

Klevay (86), using the method described by Cole et al. (84), confirmed the study of Kunze and Laug (87) that dieldrin is stored to a greater degree in the adipose tissue of female rats than of male rats. He found that the greater ability of male livers to excrete dieldrin is consistent with the greater storage by females and lesser toxicity to males.

The isolated perfused rat liver was used to study the metabolism of phthalate ester plasticizers used in plastic tubing and bags for blood storage (88). The method of Miller et al. (85) was used. The system perfused an isolated liver for four hours. The authors concluded that the plasticizer butyl glycolylbutyl phthalate is extracted from plastic tubing by the liver, and its product glycolyl phthalate is secreted into the perfusion media.

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The metabolism of drugs which are eliminated solely by biotransformation in the liver show good correlation in intact animals and in isolated perfused liver systems (89). Metabolism studies using the isolated perfused rat liver, included studies on the rate of pentabarbital disappearance from the medium of livers isolated from normal and Walker 256 tumor bearing rats (90), and the protective effect of oleate on metabolic changes produced by Halothane in rat liver (91). Livers of 48 hour fed or starved rats were perfused and exposed to Halothane (2.5% v/v). Solutions of oleate (0.1 M) were prepared in albumin (10% v/v) and added to the medium. The inhibitory effects of Halothane on O_2 consumption and urea synthesis are conteracted when oleate is added to the perfusion medium. Oleate also counteracts Halothane induced inhibition of urea synthesis and changes in liver tissue metabolite concentration. The degree of halogen induced inhibition of gluconeogenesis from lactate is also decreased by oleate.

BRAIN

The inhibitory effect of several narcotic analygesics and other psychotropic drugs on the active uptake of ^{3}H-norepinephrine was studied in mouse brain slices and synaptosomes (92). Codeine, hydromorphone, levorphanol, meperidine, methodone, morphine and naloxone inhibited the uptake of norepinephrine. The study suggests that the inhibition of the uptake of norepinephrine is related to the lipid solubility rather than the specific structures of narcotic analygesics.

Nonbarbituric hypnotics were studied in the Warburg manometric apparatus using brain slices of female Wistar rats (93). "Valmed" (ethinylcyclohexyl carbamic acid) was compared with "Seconal Sodium" (sodium allyl, 1-methylbutyl barbiturate) in its ability to inhibit the potassium-stimulated respiration of slices of brain cortex. In concentrations equivalent to those obtained during anesthesia "Seconal Sodium" depressed the oxygen uptake of normal tissue whereas both Seconal Sodium and Valmed inhibited the respiration of KCl-stimulated tissue.

Rat brain homogenates and whole brain tissue slices were used to study the toxic effect of 6-hydroxydopamine (2,4,5-trihydroxyphenyltetylamine) on nerve terminals (94). The uptake of ^{3}H-labeled dopamine, norepinephrine and serotonin into rat brain homogenates was measured. The data showed that 6-hydroxydopamine generated hydrogen peroxide and that hydrogen peroxide can damage the biogenic amine uptake system. Hydrogen peroxide generated from 6-hydroxydopamine that accumulated in catecholamine terminals may be the cause of the long-lasting catecholamine depletion that accompanies the destruction of nerve terminals. Tissue slice experiments performed with (^{3}H)-5-hydroxytryptamine produced the same results.

Soman (pinacolyl methyl phosphonofluoridate) and DFP (diiopropyl phosphorofluoridate), potent inhibitors of esterases and particularly cholinesterase, were studied in slices of cerebral cortex (95). Respiratory rates were measured by the conventional Warburg technique. The compound was added to the incubate in 0.2 ml isopropyl alcohol. Respiratory rates were depressed by Soman at concentrations between 10^{-4} M and 10^{-2} M and at concentrations of DFP between 5×10^{-3} M and 5×10^{-2} M.

The effect of hyperbaric oxygen on oxygen uptake in guinea pig cerebral cortex slices incubated in Krebs-Ringer glucose saline solution or in the presence of various substrates was studied (96). The concentrations of glycogen inorganic phosphate, phosphocreatinine, adenosine triphosphate, K^+, and Na^+ were studied with glucose as the oxicizable substrate. Formation of lipid peroxides, thought to be connected with oxygen toxicity, were measured. Tissue oxidative reactions, phosphocreatinine, adenosine triphosphate and intracellular ions were diminished and lipid peroxides increased. The increase in lipid peroxides is attributed to the toxic effects of hyperbaric oxygen.

Myelination was inhibited by 5-bromodeoxyuridine in sections of newborn rat cerebellum cultured in the Maximow double coverslip assembly (97). Explanted pieces of newborn rat cerebellum maintained in organ culture show similar morphological and biochemical maturation to *in vivo* development. Myelinated axons appear in the cultures at 10 to 11 days. When 5-bromodeoxyuridine at a concentration of 1.5×10^{-4} M was in the culture from explant, the cultures looked as healthy as controls except that few or no axons became myelinated. Thymidine at 2,5, or 7.5 times the concentration of 5-bromodeoxyuridine when added to the cultures simultaneously with 5-bromodeoxyuridine prevented the inhibition of myelination.

TRACHEA

Ciliary activity, mucous flow and muscle contractions are important mechanisms in the trachea for removing and clearing foreign particulate matter from the upper respiratory tract. Ciliary depressant action of components of cigarette smoke was studied by Kensler and Battista (98). The trachea tissue was placed in Tyrode's solution aerated with 95% O_2-5% CO_2, and sutured to a tracheal holder which raised the center portion of the trachea. Tracer particles were a mixture of finely powdered soot and lycopodium spores. The effect of materials on ciliary transport was determined by measuring the time required for the particles to move a distance of 5 mm. Bleiberg (99) in studying the combined effects of metaproterenol and cigarette smoke on ciliary activity in the rabbit trachea used a slightly different method. A humidified, temperature-controlled tissue chamber was inclined at an angle of 15°C from the horizontal with the cranial end of the tissue at the upper end of the chamber. The uphill axial movement of glass microbeads (0.03 mm in diameter) placed on the mucous surface of a section of trachea was measured as an index of the mucociliary transport activity. Readings were obtained by measuring the time in seconds for a single bead to travel a known distance as viewed against the microscope reticle at five-minute intervals.

Bleiberg (100) reported that Freon-propelled metaproterenol aerosols produced a sustained increase in muco-ciliary transport rate in rabbit tracheas. Tracheas were exposed to puffs of cigarette smoke prior to and after metaproterenol application. Pretreatment with metaproterenol increased the number of puffs of cigarette smoke required for complete inhibition of muco-ciliary transport activity. When the trachea was exposed to aerosol metaproterenol after ciliastasis from cigarette smoke, there was some recovery of transport activity.

Isolated segments of hamster and rat trachea in organ culture are used to study the effects of carcinogenic compounds on respiratory tissue. A detailed examination of the bioepithelium maintained in culture was done by Kaufman (101). Maintenance of tracheas *in vitro* allows for more extensive incorporation of labeled precursors by the tracheal epithelial cells than is possible by the administration of comparable amounts of labeled precursors *in vivo* (101). The respiratory tracts were removed *in toto* from young adult male Syrian golden hamsters 2-4 months old, and the tracheas separated from other tissue. Tracheas incubated in Lerbouitz L-15 medium, containing L-glutamine and ^{3}H-5-uridine, were examined microscopically and biochemically. The authors suggest that the interpretation of biochemical changes during and after carcinogen administration needs to be correlated with the morphologic evaluation because of the changes in cellular populations occurring in the trachea.

Crocker and Sanders (102) reported on the influence of Vitamin A and 3,7-dimethyl-2,6-octadrenal (Citral) on the toxic effect of benzo(a)-pyrene on hamster trachea in culture. They used tracheas from Syrian hamsters 2 to 4 days old. Two strips of mesh bearing explanted tracheas were laid on the surface of clotted medium in the center of an organ culture dish provided with an outer circumferential well containing a moistened filter paper. The concentration of benzo(a)pyrene was about 10.5 µg/ml (0.041 mM) in medium. The clot surrounding the mesh was removed and 0.05 ml Tyrode's solution containing tritiated thymidine was dropped on each explant at 8 or 15 days, and finally fixed in Bouin's fixative. The study showed that benzo(a)pyrene and Vitamin A act directly on the respiratory epithelium in a competitive fashion and on cartilege with an additive effect. The authors state "the organ culture method thus appears applicable to the study of the mechanisms of action of Vitamin A and a carcinogenic polycyclic hydrocarbon on trachea-bronchial tissues."

Crocker (103) exposed suckling rat trachea in organ culture to three known carcinogens: 9,10-dimethyl-1,2-benzanthracene, 3,4-benzypyrene and 20(3)-methylcholanthrene. The effects of these three compounds were similar, in that all produced an increase in the proportions of basal cells undergoing DNA synthesis. The authors compared three systems, human fetal lung, adult mouse prostate and suckling rat trachea and concluded that the unifying similarities in the three systems are suppression of mesenchyme, stimulation of basal cell replication, and induction of metaplasia.

LUNG

Piper and Vane (104) describe a method for the perfusion of isolated guinea pig lungs using Krebs solution. This new assay method was used to detect the release of active substances during anaphylaxsis in guinea pig lung. Piper and Vane found histamine "slow reacting substance-A, prostaglandins E_2 and F_2 a, and other substances not previously described released into the perfusate." The heart and lungs were removed and the pulmonary artery and trachea cannulated. The lungs were suspended in a chamber and perfused through the pulmonary artery with Krebs bicarbonate solution gassed with 95% O_2-5% CO_2 at 37°C. Lungs were taken from guinea pigs sensitized 28 days previously with ovalbumen or from unsensitized guinea pigs. The effluent from the lungs was taken to superfuse a series of isolated assay tissues; stomach strip, duodenum and colon of the rat, longitudinal strips of jejunum and terminal ileum of the cat, spirally cut strips of thoracic aorta of the rabbit, rectum of the chick, ileum and trachea of the guinea pig. The authors conclude "the antagonism by aspirin-like drugs of the release of RCS (new undescribed substance) could well provide a basis for new and relatively simple in vitro screening tests for anti-inflammatory compounds."

Shabad et al. (105) state that "the organ culture method of lungs can be used to detect precancerous conditions and to test for rapid determinations of the oncogenic activity of some chemical compounds." A modified method of Chen (113) was used for the organ culture of embryonic lungs of mice and rats. Strain A and C3HA mice and BD-1X rats were used. Strain A mice have a high incidence of mammary gland carcinomas and adenomas of lungs. Strain C3HA had few lungs adenomas. The transplacental effects of urethan, dimethylnitrosamine and nitrosomethylurea were shown. Pregnant animals were treated with these agents 15-18 days after copulation. Females were killed on days 19 and 20 and lungs of embryos removed. Pieces of embryonic lungs was explanted on cellulose plates and floated on the surface of a liquid nutrient medium. These organ cultures from intact mice and rats were maintained for 30-33 days. The effect of administration of urethane in vivo was compared with that seen in culture. Pregnant mice were treated with doses of urethane (30, 60, 90-100 mg) on days 15-18 of gestation and their offspring were examined for lung adenomas on the same days that the organ cultures were examined. Lung adenomas and precancerous changes were seen in offspring of mice treated with urethane depending on size of dose and time of observation. Similar changes in organ cultures were observed depending on size of dose except that lung adenomas developed more rapidly in vitro than in vivo. With urethane the first adenoma was recorded as early as 4 days of explantation and at 14 days adenomas were observed in two-thirds of the explants. The authors conclude "these data should be taken into consideration in working out prophylactic and hygienic measures for protection of populations from air pollution, cigarette smoke, etc. The increased sensitivity of embryonic tissue to oncogenic agents makes it necessary to give the highest priority to the protection of the health of pregnant women and newborn."

Fetal human lung tissue from 3-5 months foetuses was exposed in culture to 3:4 benzpyrene in concentrations of 1,4 and 6 µg/ml of medium (106). The addition of the carcinogen induced epithelial hyperplasia of bronchioli and pneumonomeres and inhibition of stromal growth at all concentrations. The percentage of explants showing hyperplasia was the same (84-89%) for all three concentrations of 3:4 benzpyrene but the first appearance of hyperplastic change was speeded up and the number of hyperplastic foci per explant and the degree of hyperplasia in them increased with rising concentration of the carcinogen.

O'Donnell et al. (107) reported on the maintenance of normal metaplastic and diplastic states of human bronchial mucosa in organ culture and this work was furthered (108) to study the toxicity of benzo(a)pyrene and air pollution composite for adult human bronchial mucosa in organ culture. Bronchial mucosal patches from adult humans were maintained in organ culture for 5 to 11 days. (Benzo(a)pyrene (15 µg/ml) or an air pollution composite (700 µg/ml) were present in culture media during incubation of explants. All samples were incubated with tritiated thymidine before fixation and each tissue piece was examined by histological and autoradiographic methods. Benzo(a)pyrene and air pollution composite produced toxic destruction of all cell types or as a less marked effect, suppression of DNA synthesis and distortion of morphological states of columnar but not of regenerative epithelia. Toxicity due to air pollution composite cannot be identified with either a single component of air pollution composite nor any particular cell type. Toxicity of benzo(a)pyrene was interpreted as evidence that microsomal mixed-function oxidases are present and active in metabolic conversion of benzo(a)pyrene.

Rajan et al. (109) studied the response of human pleura in organ culture to asbestos fibers. Human parietal pleura were dissected into 2-mm square pieces and maintained in organ culture in a synthetic medium containing calf serum. Blue asbestos was suspended in the medium at a concentration of 0.01%. The culture vessels were maintained in an atmosphere of 5% CO_2, 50% O_2 and 45% N_2, for up to 8 days. Explants in the presence of asbestos showed marked proliferation of mesothelial cells when compared with controls in normal medium. In some areas there was invasion of the underlying tissue by the cells and the cells had larger nuclei than controls. There was also an increased amount of collagen in the underlying tissue.

KIDNEY

Cortex slices, isolated perfused kidneys, entire papilla and isolated renal arteries have been used to study the effects of vasoactive substances, diuretics, cardiac glycosides and other compounds on renal function. Hysell and Bohr (110) used the isolated perfused rat kidney to determine renal vascular response to rat plasma, a vasoactive fraction of hog plasma, epinephrine, angiotensin, KCl, serotonin, and vasopressin. A perfusion pump maintained a constant rate of 3 ml/min of perfusate through polyethylene tubing leading to a cannula in the renal artery. An injection site just prior to the cannula in the renal artery made possible the rapid administration of small volumes of test substance. Calcium concentrations in the perfusion solution were varied--alternate perfusion fluid was whole rat plasma. Addition of rat plasma or a vasoactive fraction of hog plasma caused an increase in renal vascular resistance. The time course of the responses caused by these two agents was the same but differed from that of the pressor responses caused by epinephrine, angiotensin, KCl, serotonin or vasopressin.

Rosenfeld *et al.* (111) studied the effect of ouabain and potassium on the isolated perfused rabbit kidney. They found that plasma potassium levels as high as 12.0 meq/liter did not inhibit ouabain induced natriuresis and diuresis, whereas in animals loaded with potassium a marked inhibition did occur.

Krahe *et al.* (112) studied the action of exogenous angiotension on glomerular filtration rate and filtration faction in the isolated perfused rabbit kidney. The kidneys were perfused at a constant pressure of 97.1 ± 3.0 mm/kg with a suspension of 40% volume rabbit erythrocytes in Tyrode's solution to which 4 g % bovine albumin was added. When angiotensin II-amide in a dose of .1 $\mathrm{ng \cdot m^{-1} \cdot min^{-1}}$ was infused into the perfusion system for 10 minutes, the glomerular filtration rate and filtration factor increased during the infusion and fell to former levels after the infusion.

The method for growing embryonic kidneys in tissue culture preparations has been described by Chen (113).

Shabad *et al.* (114), using the method of Chen, studied the transplacental effect of 7/12-dimethylbenz(a)anthracene, benz(a)pyrene, and their analogs anthracene and pyrene, o-tolidine, 3,3'-dichlorobenzidine, nitrosamine o-aminoazotoluene and p-aminoazotoluene. Kidneys were obtained from 19 to 21 day old mouse embryos. The embryos were obtained from mice that during the last third of their gestation received the above compounds. Explants were fixed with Bouins fixation at 4, 7, 11, 14, 18, 22, 26 and 30 days. They showed that mouse kidney embryonic tissue can be explanted in organ culture for 3 to 4 weeks. Embryonic tissue which was subjected to transplacental treatment of the different chemicals, revealed a more intense growth of epithelium and survived longer than did control cultures.

Crocker and Vernier (115), using embryonic mouse kidneys, showed that alterations of potassium concentration was one of the most likely causes of renal maldevelopment. In order to correlate animal data with human disease, Crocker (116) used human embryonic kidneys in organ culture. Kidneys were obtained from embryos 5 to 12 weeks of gestation and cultures were grown for 2 to 5 days. Medium 199 prepared virtually free of potassium was used. Control kidneys were grown in a medium with a potassium concentration of 6.5 to 10 meq/liter while the other kidney was grown in a medium with potassium of 3 to 6 meq/liter. In 38 embryonic kidneys grown with a potassium concentration under 6 meq/liter, the following defects were seen: (1) decreased number of branches of the ureteral bud; (2) failure of nephron induction at the site of branching; and (3) occasional dilatation of the ureteral bud.

INTESTINE

Isolated segments of gastrointestinal tract provide a valuable test system for studying intestinal hydrolysis, conjugation, metabolism and transport. Pekas and Paulson (117) incubated everted sacs of rat small intestine in a pH 7.4 media containing either the insecticide 1-(1-^{14}C) naphthyl N-methylcarbamate (carbaryl), or 1-(1-^{14}C) naphthol, and isolated from mucosal and serosal fluids and the metabolite 1-(1-^{14}C) naphthyl glucuronide. The hydrolysis of carbaryl and conjugation of naphthol indicated some degree of metabolism by the intestine before absorption. Pekas (118, 117) used the method of Wilson and Wiseman (119). The small intestine of young rats extending from the bile duct to the cecum was divided into approximately equal parts and each section was everted and incubated for two hours with 10^{-5} M carbaryl at 37°C under an atmosphere of 95% O_2-5% CO_2. The ^{14}C labeled constituents were chromatographed on silica gel thin-layer plates, and the radiospots scraped and counted by liquid scintillation.

Shaw and Guthrie (120) studied penetration through isolated sections of the mouse gastrointestinal tract of five insecticides; malathion, carbaryl, dimethoate, DDT and dieldrin. To study the effect of age on penetration, sections of small intestine and sections from the colon and anterior region of the rectum were obtained from mice 12 days, 6 weeks and 12 weeks old. Solutions of radioactive insecticide (0.25 ml) were introduced into the lumen. After 80 minutes the amount of radioactivity in the serosal and luminal solutions and that remaining in the tissue were determined. The extent of penetration for mice of different ages showed only trivial differences for carbaryl, DDT and dieldrin but both malathion and dimethoate penetrated more rapidly through tissues of 12 day old mice. Generally the amount of insecticide bound in gut tissues was greater in 6 and 12 week old mice than in 12 day old mice. DDT and dieldrin were exceptions. The authors found that differences in absorption of insecticide may occur in specific regions of the digestive tract. Phosphate and carbamate insecticides, but not chlorinated hydrocarbons, showed greater penetration into the colon.

Stookey _et al_. (121), using the method of Wilson and Crane (122), studied fluoride absorption using intestinal segments from young rats which were maintained on low fluoride diet and fluorine-free water. When four different levels of fluoride were placed inside the intestinal segments and rate of diffusion of fluoride through the intestinal wall measured, comparable values were obtained, suggesting that the amount of fluoride present is not a major factor governing the rate of diffusion. The authors found that the rate of absorption through the intestinal wall was about twice as great as in the stomach and that the rate of absorption through the intestinal wall is related to the surface area of the intestine. They presented evidence that an active transport system is not involved in absorption of fluoride in the rat.

Moore _et al_. (123) studied the effect of synthetic surfactants, alkylbenzenesulfonate, linear alkyl sulfonate, cetyltrimethylammonium bromide and Triton X-100^{6} on intestinal permeability to glucose in the absence of a functioning active transport mechanism. Phloridzin was used to block the active transport of glucose. Segments of the small intestine of the golden hamster were used. The surfactants increased intestinal permeability to glucose in a dose-related manner with linear alkyl sulfonate producing this effect at a lower concentration than any of the others tested. Microscopic examination of the intestinal segments showed that the mucosal epithelium was not altered by low surfactant concentrations which increased intestinal permeability.

Using everted segments of rat stomach and small intestine, Lasagna's group studied the metabolism of L-3,4-dihydroxyphenylalanine (L-dopa) (124). The tissues were separately incubated with ^{14}C L-dopa in Krebs-Ringer phosphate buffer. Metabolites in the tissue and in the mucosal and serosal fluids were separated by ion-exchange chromatography and the radioactivity determined by liquid scintillation counting. The metabolites found were phenylcarboxylic acid, dopamine, and other catecholamines. The authors suggested that L-dopa could be significantly metabolized in the gastric mucosa prior to absorption. The authors conclude "the everted sac preparation is simple and gives reproducible results. It not only allows the simultaneous study of drug metabolism and drug transport across gastric and intestinal mucosa, but can also provide information on the effect of drug metabolites on membrane transport of the drug."

Boass and Wilson (125) used one gram sacs of monkey intestine to study absorption of Co^{57}-labeled Vitamin B_{12}. They found that intrinsic factor was necessary for the uptake of B_{12} and that the site of maximal uptake was in the low ileum.

Using everted jejunal intestinal segments of young rats, Feldman and Gibalei (126) showed that physiologic concentrations of the conjugated bile salt, sodium taurodeoxycholate markedly increased the permeability of the everted rat small intestine to salicylate ion. Addition of egg lecithin or oleic acid and glyceryl monooleate to the medium diminished this effect and prevented changes in gross appearance of the mucosa which occurred when the isolated intestine was exposed to the bile salts alone.

The frog gastric mucosa (commonly that of Rana pipiens) is used to study gastric acid secretion isolated from the influences of blood flow, nervous factors and humoral substances (127). The method of Rehm (128) allows the study of electrophysical parameters such as H^+ secretory rate, transmucosal potential difference and the transmucosal resistance. The stomach is removed from a frog and opened along the lesser curvature, the fundic mucosa separated from the muscular layer of the stomach, and the intact mucosal membrane mounted between two Lucite chambers. The H^+ secretory rate is measured by a recording pH-stat autoburette titrator.

Nakajima (127) determined the effects of nicotine on the H^+ secretion, transmucosal potential difference and resistance using this system. The author found that nicotine produced a reversible dose-related inhibition of H^+ secretion with an increase in transmembrane potential difference and calculated short-circuit current. He suggests that nicotine may selectively inhibit H^+ secretory mechanism.

Schwart and MacKrell (129) studied the potency of an anaesthesia agent compound 347 (Ethrane - CHF_2-O-CF_2-CHF Cl) to inhibit frog gastric secretion. It was previously shown that the percentage of anaesthetic required to produce a decrease in the H^+ secretory rate in frog is proportional to the minimal anaesthetic concentration in man (the amount required to prevent a muscular response to a skin incision in 50% of subjects to whom the anaesthetic is administered). Ethrane was compared with methoxyflurane, chloroform, halothane, and flurooxene. Their results suggest lipid solubility as a factor influencing the potency of anaesthetics in inhibiting acid secretion.

Similar methods and measurements were made by Dinno et al. to study the potency of barbiturates to inhibit frog gastric section (13). Barbiturates were added to the nutrient solution to yield 1 mM concentration of barbital, diallylbarbituric acid, phenobarbital, pentobarbital, secobarbital or thiamytal. Insofar as the hypnotic activity of narcotics such as barbiturates is closely related to their relative lipophilic character as defined by the logarithm of the octanol water partition coefficient log P, the authors determined to what extent lipid solubility of barbiturates, as defined by log P, is a factor in inhibiting frog gastric secretion. They found that the decreases in H^+ secretory rate in frog gastric mucosa was almost directly proportional to log P, up to an 80% decrease in H^+ secretion rate.

Embryonic chick intestine maintained in organ culture has been used to study the effects of Vitamin D_3 on calcium transport (60, 131) and the effect of hydrocortisone on enzyme induction (132, N.Y. Academy ref., PSEB, Dec. 1966).

The method for preparing embryonic chick duodenum in tissue culture was described by McCarty et al. (133). The duodenal loop of 14 to 20 day old embryonic chicks was removed, sectioned transversely into 1 to 1.5 mm fragments, and the fragments arranged with the villi upward and the serosal surface contacting a Millipore membrane. The tissue and membrane were floated over Eagle's medium and incubated under a drop of medium in a humidified chamber.

Hijmans and McCarty (132) used half of a duodenum as control and added 0.5 μg hydrocortisone to the other half in a culture vessel. They found that embryonic duodenal cultures repsond to hydrocortisone with an increase in the specific activity of invertase, and that this response is dependent on the age of the initial tissue explant, duration of exposure to hydrocortisone and the pretreatment of the tissue before hydrocortisone application.

In further studying the effect of hydrocortisone on 16 and 19 day old chick duodenum, Hijmans and McCarty (132) used a chemically defined medium. They found that embryonic chick duodenum could be cultured for at least three days in a chemically defined serum-free medium. Cells in mitosis were present in cultures with or without serum. Invertase activity increased in cultures without serum and the induction of invertase by hydrocortisone is greater in serum-free medium.

Corradino and Wasserman (60) used embryonic chick duodenum to study Vitamin D induction of a calcium-binding protein in the intestine. Crystalline Vitamin D_3 added to the culture medium (400 IU per milliliter of medium) induced the formation of a substance immunologically identical to chick intestinal calcium binding protein, and enhanced the uptake of radiocalcium by the intestine. The chick intestine does not normally produce calcium binding protein until after hatching.

Corradino (131) studied the response of the chick duodenum in organ culture to the metabolites of Vitamin D_3, 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol. The authors state that there is a marked similarity between responses to Vitamin D in vivo and in this system, and that the mode of action can validly be studied in organ culture. They suggest that it might be possible to utilize the extremely high potency of 1,25-dihydroxycholecalciferol in this culture system as the basis for a simple yet highly sensitive bioassay which might be used in studying normal and disordered states of calcium metabolism.

ADRENAL GLAND

Systems for investigating the action of chemicals upon adrenals have included isolated perfused adrenals (cow 134, 135; dog 136), cortex slices (cow 137), and portions of whole adrenal (rat 138, 139, 140).

Hart and Straw (136) studied the effect of o,p'-DDD on ACTH-induced steroid production. The isolated dog adrenal was retrogradely perfused for a one hour period with 2,2'bis-(2-chlorophenyl, 4-chlorophenyl)-1,1-dichloroethane (o,p'-DDD). In adrenals perfused with plasma containing a maximum stimulating concentration of ACTH and o,p'-DDD, the response to ACTH was almost completely blocked. Steroid production fell at approximately the same rate as in the intact dog receiving an intravenous injection of 60 mg/kg of o,p'-DDD. o,p'-DDD inhibited ACTH-induced steroid production by greater than 90% within 2 hours. A 30-minute control perfusion with Krebs-Ringer bicarbonate measured baseline steroid production. This was followed by a 3-5 minute perfusion with ACTH and another 30-minute control period when the steroid response to ACTH was monitored. The adrenal was then perfused for 2 hours with either Krebs-Ringer bicarbonate or control plasma containing either drug solvent or o,p'-DDD.

By this method a statistically significant correlation of the in vivo action of o,p'-DDD in the intact dog and in the isolated perfused dog adrenal was demonstrated. When Krebs-Ringer bicarbonate glucose was used as the perfusion medium instead of plasma, ACTH-induced steroid production was not inhibited. However, when o,p'-DDD is introduced into adrenal slice preparations, the response to ACTH is not blocked and this is attributed to lack of penetration to the interior of the slice.

Perfusion of cow adrenals with acetaldehyde (100 μg/ml) resulted in 1,3,3,4-tetrahydroisoquinoline alkaloids being formed (135). Acetaldehyde reacts with tissue catecholamines, epinephrine, and norepinephrine to form these alkaloids. Insofar as ethanol is oxidized to acetaldehyde and is known to stimulate adrenocortical secretions in both man and animals, the authors suggest that these alkaloids are either secreted or leaked from nerve termini and contribute to behavioral changes caused by ethanol. The metabolites were measured through TLC of tissue homogenate supernatants.

The lowest acetaldehyde concentration at which 1,2,3,4-tetrahydroisoquinolines occurred in perfused cow adrenals was over 100 times that which results in the blood of man ingesting a moderate amount of ethanol. The authors feel that this limitation was due to current technology related to merging of catecholamines on chromatograms.

Beef adrenal cortex slices were used to further characterize the effects of ethanol on steroid synthesis (137). One and 2% ethanol inhibited aldosterone synthesis presumably at the site of conversion of corticosterone to aldosterone. The authors suggest that this method provides a means of examining specific actions of various agents directly upon the adrenal without the modifying effects of pituitary and other systems. However, the level of ethanol used was much higher than that reached in severe intoxication in man.

HEART

Isolated atria, perfused hearts, ventricles, aortic strips and embryonic hearts have been used to study the effects of chemicals on the cardiovascular system. Increased contractile force (a positive inotropic action) rate, atrial fibrillation, metabolism, and ion transport are among the effects studied, using cardiac glycosides, steroids, prostaglandins and other drugs.

To study the effect of aldosterone on ouabain-induced potassium loss, left atrial tissue, obtained from the hearts of young rabbits was perfused with a Ringer solution (142). The atria was electrically stimulated at a contract rate of 200 beats per minute and measurements made of isometric contractile tension, effective refractory period, contracture and electrical excitability. After a period of equilibration, drug effects were studied by changing over to a perfusion medium containing a known concentration of ouabain, d-aldosterone or a combination of both; d-aldosterone did not prevent potassium loss caused by a high concentration of ouabain.

Levy _et_ _al_. (142, 143 and 144) used isolated rabbit left atria to study the effect of d-aldosterone on ouabain-induced potassium loss (142) and the Na or K content of stimulated cardiac tissue (142) and the effects of Prostaglandin E_2 on isolated cardiac tissue (144).

D-aldosterone was found to be ineffective in preventing K^+ loss from isolated atrial tissue caused by high concentrations of ouabain. Also there was a lack of antagonism of ouabain-induced changes in contractile and electrical properties of atrial muscle. This effect is in contrast to the intact animal. In vivo aldosterone enhances K accumulation in skeletal muscle. In the isolated heart d-aldosterone did not promote K accumulation.

Prostaglandin E_2 was studied for its effects on the contractile force of electrically driven or spontaneously beating rabbit atrial preparations (144). Prostaglandin E_2 produced a reproducible biphasic inotropic action on rabbit atrial tissue. Other prostaglandins were reported to produce a positive inotropic effect in vivo and in vitro.

Isolated rat atrium was used to study myocardial utilization of metabolic substrates required for tissue energy production and muscle contraction. The influence of the medium which is in contact with the atrium has been studied by Paradise et al. (145, 146, 147), Venturi (148), and Lacuara (149). Citrate bicarbonate-free medium and halothane produced rapid depression of atrial contractility. Glucose added to depressed atria produced a marked increase in the force of contraction (147).

Talesnik and Sunahara (150) used the Langendorff method for isolated perfused rat hearts to study the effect of aspirin like substances as suppressors of prostaglandin inhibition of metabolically-induced coronary vasodilation. The authors postulated that an inhibitor of prostaglandin synthesis would be useful in preventing coronary insufficiency in conditions of cardiac stress. They investigated the action of indomethacin and aspirin on the metabolically induced coronary vasodilation which follows the increase of cardiac activity produced by noradrenaline Ca^{2+} and tachycardia. Young male rats were anaesthetized with ether and the hearts isolated and perfused with Krebs-Henseleit bicarbonate perfusate. The perfusate contained one half the usual calcium concentration. Phentolamine was added to the perfusate to inhibit noradrenaline. The force of contraction and coronary flow were measured continuously. Aspirin and indomethacin enhanced metabolically induced coronoary vasodilation. Cardiac hyperactivity produced by noradrenaline, Ca^{2+} or tachycardia was not stopped.

Isolated rat heart treated with 100 µg of the antibiotic chlortetracycline followed by 1 µg epinephrine developed both inotropic and chronotropic irregularities (151). The authors postulated that this effect is caused by chelation of the co-factor of cholinesterase which inactivates the enzyme and permits acetycholine to accumulate and exert its inhibitory effect on the heart.

Vahouny et al. studied factors affecting fatty acid synthesis, oxidation and esterification using the intact perfused rat heart (152, 153, 154, 155). Puramycin, an inhibitor of protein synthesis, was shown to have a direct effect on the Krebs cycle activity by using aspartate $1\text{-}^{14}C$ an immediate precursor of oxalacetate, an intermediate in the Krebs cycle (155).

An isolated perfused working heart from young female rats was used by Hamberger and Isaksson to study the effect of chloramphenicol on mitochondrial and extramitochondrial systems (156). The authors believed the isolated perfused working heart is advantageous for investigations of metabolic inhibitors on protein synthesis in the cell, insofar as increased load on the heart causes higher rates of protein synthesis. In this method developed by Morgan, in contrast to the Langendorff method, the heart does the work. The perfusate is introduced into the left atrium and pumped by the left ventricle against a hydrostatic pressure head. Chloramphenicol was introduced into the perfusate at concentrations of 50, 100, 250, and 500 µg/ml perfusate. Chloramphenicol decreased mechanical performance of the heart and caused a marked reduction in glucose uptake and lactate production. Chloramphenicol inhibition of mitochondrial protein synthesis in the isolated heart was approximately 70%.

Isolated perfused rabbit hearts, using various modifications of the Langendorff technique, were used to further investigate ionic alterations after irradiation (157, 158) and effects of fatty acids (159) on the cardiovascular system. Isolated rabbit hearts were irradiated at the rate of 500 r per minute and compared with dog hearts irradiated at 150 r per minute. The magnitude of the loss of potassium and the radiation dose producing it were approximately the same for both systems. The loss of potassium from hearts following cardiac arrest and further loss occurring after resuscitation may be responsible for arrhythmias noted in some human hearts following revival (157). The loss of potassium from arrested and resuscitated rabbit hearts paralleled the potassium loss observed in congestive heart failure in man (157). Tissue calcium was increased in the isolated rabbit heart after resuscitation and this was in agreement with work reported in the dog.

Connor et al. (159) studied the effect of both saturated and unsaturated fatty acids in the isolated rabbit heart. 0.1% stearic acid or 0.1% oleic acid solution was added to the perfusate to the coronary vessels. These fatty acids were very toxic; the coronary flow and the rate and amplitude of contractions progressively deteriorated until there was death of the heart. Equimolar albumin incubated with the fatty acid solutions bound the fatty acids and prevented this toxic effect. Unbound fatty acids, saturated or unsaturated, were extremely toxic to the heart.

Mir et al. (160) used the isolated perfused rabbit heart to study the pharmacological and toxicological effects of methacrylate monomers on cardiac function. They recorded the effects on the cardiac rate per minute, force of contraction and coronary flow (ml/min), of 13 methacrylate monomers each at dilutions of 1:1,000, 1:10,000, and 1:100,000 in Locke's solution. These compounds showed marked effects upon the isolated heart. Dose-response data are presented based upon both molar concentrations and volume dilutions of these compounds.

The perfusion method of Langendorff with various modifications has also been applied to the isolated feline heart. Swaine (161) studied the effect of phenoxybenzamine on Tyramine-induced catecholamine release in the isolated perfused cat heart. After perfusing hearts for one-half hour with Krebs-Henseleit solution containing phenoxybenzamine, they were perfused with phenoxybenzamine-free Krebs-Henseleit solution before addition of Tyramine to the perfusate. The effect on the isolated cat heart by a muscle relaxant 2-amino-5-phenyl-1,3,4-oxadiazole hydrochloride caused no significant effect (162).

Tanz et al. (163) studied 9-alpha-fluorohydro-cortisone in the isolated feline heart as well as the cat papillary muscle. The findings of a positive inotropic effect in low concentrations (0.5-1.0 µg/ml) and a negative effect in higher concentrations (10-20 µg ml) correlated with histologic changes in cardiac muscle and with intramuscular ion exchange (163).

Embryonic hearts are a valuable system for studying oxygen consumption, cell permeability and drug effects (164, 165). Chick embryo hearts have been used at varying ages of the embryo and sensitivity to drug effects at the different ages of development compared. The effects of tetrodotoxin on chick embryo hearts, single chick heart cells and aggregates of chick heart cells were compared (165). Hearts were dissected from chick embryos aged 2 to 12 days. Tetrodotoxin is reported to abolish spontaneous activity in cells whose action potential is dependent on a transient increase in sodium conductance and in the chick heart this mechanism functions. Sensitivity to tetrodotoxin increased with increasing embryo age. All hearts from 2-4 day old embryos continued beating; by day 7, 43% of the hearts stopped beating. Only a small fraction of isolated cells are sensitive to tetrodotoxin whereas aggregates formed from single cells are similar to intact heart in both age-related and dose-related sensitivity (165). Chick embryo hearts are useful for studies of glucose transport and insulin action (164). It was found that 2-deoxyglucose inhibits glucose uptake by 5 and 9 day old chick hearts and this inhibition is not modified by insulin action. The mechanism regulating glucose uptake develops between the seventh and tenth day of embryologic development in the chick heart and at this stage glucose transport limits glucose uptake (164).

Recent developments in maintaining fetal hearts in organ culture provide a system for studying the toxicity of compounds at various stages of fetal development as well as a potential bioassay for cardioactive drugs. Both intact fetal hearts and slabs of fetal hearts have been maintained in culture. Hughes and Longmore (166) studied stages of development of mouse fetal hearts to survival time. Heart rates were measured at different intervals during the culture period. The authors also studied the beating and survival time for human, dog, cat and rabbit fetal hearts. Mouse fetal hearts were cultured, on steel grids, from newborn, and 12-21 day old mice.

Nineteen-day old fetal mouse hearts were beating and survived for 152 days. In comparing survival time of different species of different ages, it was found that younger and smaller fetal hearts survived the longest time. Slabs of tissue survived for shorter times than did intact hearts.

The performance of composite fetal hearts was studied (165). Hearts from litter mates, from different species and at different stages of development were used. After 144 hours composite hearts beat synchronously. The beating rate of the composite was the rate of the faster part. Intact hearts from 38% term fetal cats survived for 39 days; 66% term fetal rabbits survived 15 days.

BONE

A test system using fetal rat bones in tissue culture was used to study agents that stimulate bone resorption. 25-hydroxycholecalciferol (25-HCC) (168), 1,25-dihydroxycholecalciferol (1,25-DHCC), and endotoxin (169) were among the agents tested.

In this method (170) bone shafts from 19-day rat fetuses labeled with ^{45}Ca *in vivo* were incubated for 24 hours in a chemically defined medium supplemented with 5% human serum inactivated at 60°C for 30 minutes. Paired bones were transferred to vessels containing the same medium with or without the test material.

Vitamin D_3 is hydroxylated in the liver to produce 25-hydroxycholecalciferol (25-HCC) which then enters the circulation and is taken up in target organs (176). 25-HCC is further converted to other active metabolites by a second hydroxylation largely in the kidney. One of these metabolites is 1,25-DHCC which is more potent than 25-HCC in mobilizing calcium *in vitro* but not *in vivo*. The efficacy of these materials in treating richetic animals was determined. In the *in vitro* system the release of previously incorporated 45 calcium from bones was measured. Endotoxins (169) were also used to stimulate release of 45 calcium from fetal rat bone in tissue culture. Endotoxins may play a role in bone loss characteristic of human periodontal disease.

A test system utilizing 3 to 6 day old mouse calvaria in tissue culture was used to study parathyroid (172) as a powerful inducer of bone resorption, and diphosphonates (173) and 2-thiophenecarboxylic acid (174) as inhibitors of bone resorption. To study the inhibitory effect of 2-thiophene-carboxylic acid, Fang *et al*. (174), used cavaria of Swiss albino mice of the Webster strain aged 4 to 6 days. The frontal and parietal bones were removed aseptically and cultured by two different procedures. In one method, the bone was attached to a coverslip with a mixture of chicken plasma and chicken embryo extract (2:1). Control medium was Gey's balanced salt solution and heated horse serum (1:4). In this control medium, a net uptake of calcium into bone was observed. Bones were cultured in this medium containing parathyroid hormone alone and also in combination with 2-thiophenecarboxylic acid. In the second culture method, bones were first labeled with ^{45}Ca by injection of neonatal mice 2 days after birth; the bones were used 4 days later. Each calvarium was divided in half; one half served as an experimental bone, the other as the control in a paired system. The bones were cultured in a synthetic medium containing 5% heated rabbit serum. The release of ^{45}Ca from bones was measured in samples of medium by means of liquid scintillation spectrometry. The bones were dissolved in 0.5 ml formic acid and analyzed for the remaining isotope. The author concludes "2-thiophenecarboxylic acid is a simple compound, and therefore may provide a useful new pharmacological tool for studies of bone metabolism and calcium transfer."

The effect on rat bone marrow metabolism by anaesthetic agents, nitrous oxide, halothane, and cyclopropane (175) in the Warburg apparatus was studied. These anaesthetic agents are known to depress bone marrow hematopoiesis in vivo and comparable doses to that used in man were tested in the in vitro system. The measurement of the effect of these agents on rat bone marrow oxygen consumption and anaerobic glycosis showed no demonstrable inhibition at clinical concentrations. At higher concentration halothane depressed oxygen consumption.

SKIN

The skin is one of the largest organs of the body and assumes many complex functions. Among the functions studied using isolated segments of skin from different species include transport characteristics (176), endogenous respiration (177), regulatory mechanism of lipid synthesis (178), tensile strength (179), desquamation (180), and morphogenesis in organ culture (181).

Early studies utilized skin pouches from skin removed from the hind legs of frogs (176, 182). The influence of steroids upon osmotic pressure changes in skin pouches was studied (182). Ringer's solution was introduced into the pouch and the pouches were immersed in a bath of Ringer's solution. Steroids, dl-aldosterone, 2-methyl-9-α-fluorohydrocortone, prednisone, and prednisolone were added to the contents of one pouch, the other pouch served as control. dl-Aldosterone and 2-methyl-9-α-fluorohydrocortone produced an increase of osmolarity and increase in total fluid volume inside the pouch. Helman and Miller (182) reported an in vitro method for the study of frog skins that does not produce edge damage, a problem associated with skin studies. By their technique the tissue adhesive isobutyl-2-cyanocrylate (ethicon, Inc.) was used to glue frog abdominal skin to Lucite gaskets which were sealed in Lucite chambers with liquid Sylgard 184 (Dow Corning). After the tissue adhesive made contact with the skin it polymerized to form a bond between the skin and the gaskets. To determine the electrical resistance of the skin, constant-current pulses, several hundred milliseconds in duration, were passed through skins bathed on both sides with Ringer's solution. Polyethylene bridges filled with 3M NaCl-agar connected the bathing solutions to the external electronics and the tips of the voltage probes were permanently fixed 0.5 mm from the surface of the skin.

Voute et al. (180) found that aldosterone promotes a moult in isolated frog skin and this moult was associated with bioelectric changes. After addition of aldosterone, the mitochondria-rich cells become pear shaped and separate from the stratum corneum by a space filled with amorphous material called a "lake." The average number of mitochondria-rich cells with a "lake" in the outermost layer of the stratum granulosum is 9.3 in aldosterone-treated skins, whereas it is 4.0 in controls.

Ziboii and Bradi (178) used isolated segments of rat skin to study the effect of tetrolyl-pantetheine on lipid synthesis. Skin specimens (60-80 mg) were removed from the shaved and clipped back of a Sprague-Dawley rat. The specimens were incubated with a tracer amount of sodium acetate 1-^{14}C in Krebs-Ringer phosphate buffer and various concentrations of tetrol-pantetheine at 37^{o}C. To estimate the total ^{14}C incorporated into lipids, the incubation mixture was extracted four times and the radioactive acetate in the extract was removed by chromatography. The incorporation of ^{14}C into lipids from acetate 1-^{14}C was approximately 70% inhibited when tetrylyl-pantetheine was added to the incubation medium. Incubation of skin specimens with sulfhydryl compounds such as L-cysteine and 2-mercaptoethanol failed to reverse the inhibition of lipid synthesis by tetrolyl-pantetheine. The authors conclude "this study has further demonstrated the usefulness of whole skin preparations for the study of regulatory mechanisms of lipid synthesis . . ."

The morphogenetic effects of high levels of Vitamin A on adult mammalian epidermis in culture was reported by Barnett and Szabo (181). Explants of skin consisting of the superficial dermis and whole epidermis were obtained from the dorsum of the ear of adult guinea pigs and cultured with the epidermis upward using a modified raft technique. The cultures contain Eagles or BGJ medium and 22-30 IU Vitamin A alcohol dissolved in ethanol. At the time of explantation and after 3, 6, and 10 days of culture, explants were fixed and studied with an electron microscope. Marked differences from control cultures were observed. Among the changes observed were no signs of overall keratinization by 6 days *in vitro*, infrequent and short desmosomes and formation of numerous regularly spaced micro-villi on both the free and internal cell surfaces. Intercellular spaces were widened and canaliculi-like structures were formed. The authors compare their findings with those on *in vivo* squamous metalasia of adult tracheal epithelium in Vitamin A deficient rats and conclude "our epithelial cells, transforming from a keratenizing type to a mucous type, share a great many features with tracheal cells which are midway along the path of squamous metaplasia from a respiratory type."

STRIATED MUSCLE

A striated frog muscle, the sartorius, has proved to be a useful tool for studying the effects of drugs on muscle contracture, metabolism, structure, and action of the neuromuscular junction. Caffeine penetrates muscle fiber membranes and activates contractions by producing an increase in the intracellular Ca^{+} concentration (184) and is a useful reference drug for studying muscle contractions and kinetics.

Using the single neuromuscular junctions of the frog sartorius muscle, Longnecker *et al*. found that black widow spider venom causes exhaustion of miniature end plate activity and depletes the nerve terminal of vesicles (185). A sartorius muscle, with its nerve intact, was dissected from Rana pipiens and mounted in a "lucite" chamber at 20-25°C. Muscle twitch was blocked by bathing the preparation in a solution containing 0.5 M $CaCl_2$, 4 mM $MgCl_2$, 2.5 mM KCl, 110 mM NaCl and 5 mM Tris-buffer (pH 7.4). Intracellular records of end plate potential and miniature end plate potentials were obtained by impaling the end plate region of surface muscle fibers with glass micropipettes filled with 3 M KCl. Extracellular records of the nerve terminal spike and miniature end plate potentials were made with micropipettes filled with 4 M NaCl.

Marco and Nastuk (184) found that caffeine (0.25 to 2 mM/liter) produced sarcomeric oxcillations which appeared at both the neuromuscular junction and non-junctional regions of frog skeletal muscle fibers.

Feinstein and Paimre (186) present a discussion of the action of local anaesthetics on excitation-contraction coupling in both striated and smooth muscle. They report that respiratory stimulation accompanies the action of caffeine in frog muscle and is abolished by anaesthetics when the associated intracellular calcium release is blocked.

Single muscle fibers, isolated from the semitendinosus muscle of the frog, were used to study the relationship between the length of a frog striated muscle fiber and the force it can produce (187). The authors report that one of the factors responsible for decreasing the force of muscle contraction with shortening is inactivation of the myofibrils in the core of a fiber, and that caffeine antagonizes this inactivation and changes the length-force relationship at short muscle lengths.

Mouse or rat diaphragm tissue is a striated muscle which has been used to study the effects of drugs on carbohydrate metabolism and on muscle tension.

A useful modification is the stimulatory effect of insulin on glucose uptake in diaphragm tissue. Oyama and Grant (188, 189) used pooled mouse hemidiaphragms of male albino mice weighing 25-30 grams. The tissues were incubated in a Warburg apparatus in Krebs-Ringer bicarbonate, with insulin and glucose. Glucose uptake was calculated as mg glucose per 10 grams of dry weight of tissue.

In comparing the effects of phenethyldiguanide, a hypoglycemic agent, in the total guinea pig, in guinea pig liver slices and in the isolated rat diaphragm, Tyberghun and Williams (190) showed consistent results with increased glucose uptake, decreased glycogen deposition and oxygen consumption and increased lactic acid formation. The rat diaphragm was used to study cellular enzyme loss from muscle, by inducing such loss with disodium dihydrogen ethylene diamine tetraacetate (Na_2H_2EDTA) (191). The effects of ouabain on the isometric twitch tension in muscle was studied with the isolated phrenic nerve diaphragm preparation (192). Ouabain increased the isometric twitch tension of the indirectly stimulated rat diaphragm. The diaphragm was analyzed for sodium, potassium and calcium following exposure to ouabain plus indirect stimulation, indirect stimulation alone, and to ouabain alone. The greatest shifts in Na, K, and Ca occurred in stimulated muscle in the presence of ouabain (192).

A striated muscle, the chick skeletal muscle, was used to study the effects of chloroquine in tissue culture (193). Long-term use of chloroquine, in humans, for the treatment of malaria, has been associated with retinopathy and myopathy. Concentrations greater than 5 µg/ml in the culture medium interfered with myogenesis, the degree of damage varying with drug concentration and duration of exposure. The method of Nameroff and Holtzer was used (194). Mononuclear cell preparations were made, through trypsinization, centrifugation, and dissociation, from 11 day old chick embryo breast muscle tissue. Chloroquine phosphate was added to the experimental cultures before the cells were placed in petri dishes. The authors related their findings to human and animal studies and concluded that long-term therapy in which chloroquine concentrations in muscle may reach 40-80 times plasma levels would far exceed the concentrations that produced tissue culture morphologic changes.

Cohen and Fischbach (195), using muscle fibers grown from myoblasts obtained from embryonic chick breast tissue, showed that muscle fibers in 7 to 10 day cultures generate action potentials and twitch in response to depolarizing stimulus. Many fibers twitch spontaneously but the amount of activity varies from culture to culture and within a single culture over long periods of time. They demonstrated that the activity of muscle fibers that develop *in vitro* from isolated myoblasts, is sufficient to reduce the sensitivity of the surface membrane to acetylcholine.

SMOOTH MUSCLE

The isolated rabbit aortic strip is a classical assay method used to study the effects of various agents against epinephrin-induced contractions on vascular smooth muscle. The assay method of Furthgott and Bhadrakom and a modification by Wurzel *et al*. (196) uses spirally cut strips of rabbit aorta 2.0-2.5 cm in length and 2.0-4.0 mm wide. The strips are suspended in an organ bath at 37°C, in Krebs-bicarbonate solution containing glucose and continually gassed at 95% O_2-5% CO_2. The drug under test is added to the tissue bath. Tension is measured by a force-displacement trnsducer and recorded on a polygraph.

Lyons and Swain (197) used a tension of 4 g on aorta strips and 1 g on vena cava strips, to study the effects of catecholamines and the response of blocking agents to these catecholamines on both aorta and vena cava strips. Epinephrine, norepinephrine and isoproterenol contract isolated strip of both aorta and vena cava. Isoproterenol is the least potent. Phenoxybenzamine reduced contractions but they were not significantly reduced by pronethanol.

Shemano and Fallon (198) studied the effects of L-3,3'5-Triiodothyronine on epinephrine-induced contractions of isolated rabbit aorta, and found potentiation when copper acetate or trace metal contaminants were present. EDTA similarly potentiated epinephrine contractions.

St. Clair and Loflana (199) describe an organ culture method for maintaining arterial tissue in a metabolically active state for up to nine days. Segments of aorta, weighing approximately 25 mg, were obtained from normal and atherosclerotic white carneau pigeons. These segments were placed intimal surface down on grids in organ culture dishes with medium. The culture medium contained Eagle's MEM (Earles), 20-50% serum to which has been added the radioactive lipids, cholesterol 1,2,-^{3}14 and oleic acid 1-^{14}C, penicillin, l-glutamine, and streptomycin sulfate.

The amount of free and esterified cholesterol in individual segments was determined by gas-liquid chromatography following separation on TLC. Using this technique the authors demonstrated the esterification by arterial tissue of radioactive cholesterol added to the culture medium, and the effect of atherosclerosis on cholesterol esterification.

The isolated rabbit or guinea pig ileum is used to study the spontaneous activity of the intestine and the effect of measurable amounts of drugs against this activity. Mir et al. (200) studied the response of methacrylate monomers on guinea pig ileum and upon acetylcholine and barium chloride induced contractions. The spontaneous contractile activity of a 1.5-2 cm section of guinea pig ileum in Tyrode's solution was recorded, and then the test compound was added to the bath. Eight of nine methacrylate monomers tested produced inhibition of pendular movements and relaxation of the muscle within 15-30 seconds. The effect of each compound could be terminated by promptly washing with fresh Tyrode's solution. The isolated guinea pig ileum has been used widely in studying drug effect for antispasmodics, analygesics, vasoconstrictors, etc. It was used to determine the antiacetylcholine activity of d-, l- and dl-piperidyl methadone.

Ross et al. (202) found that atropine and morphine effectively block angiotensin and nicotine-induced spasm on the isolated guinea pig ileum. Hershberger and Hansen (203) found that hydrocortisone at 1.25 mg/ml inhibited effects on activated anaphylatoxin on isolated guinea pig ileum.

The uterus or oviduct is used to study effects on smooth muscle outside the gastrointestinal tract. Costa (204) studied the reactivity of uteri of spayed rats brought into oestrus by injection of ovarian hormone, to seratonin, creatinine sulfate, acetylcholine and oxytocin before and after treatment with Frenquel (alpha-4 piperidyl diphenyl carbinol hydrochloride), chlorpromazine (10-(3-diethylaminopropyl), 2-chlorophenotenothiazine hydrochloride), reserpine, demethoxy reserpine, Lergigan (N-(2-dimethylamino-2-methyl-1-ethyl) phenothiazine hydrochloride), mescaline (3,4,5-trimethoxyphenyl ethylamine sulfate) and LSD (d-lysergic acid diethylamide). They determined the uterine reactivity to a series of increasing concentrations of serotonin, acetylcholine and oxytocin starting from a minimally active concentration and increasing until the maximal uterine contraction was reached. Then the reactivity of the uterus to the contractile agents was again tested after the hallucinogenic or tranquilizing drugs had been left in contact with the uterus for three to five minutes. Frenquel, chlorpromazine, and reserpine antagonized serotonin-induced contractions but not acetylcholine or oxytocin contractions.

Mescaline facilitates serotonin activity and at high concentration causes contractions. A concentration of 1 mg/l of LSD antagonizes both serotonin and mescaline-induced contractions whereas low concentrations of LSD facilitate the effects of serotonin and mescaline on the uterus.

Lener _et_ _al_. (205) studied the relative effects of prostaglandins PGE_2 and PGF_2 on adenyl cyclase activity of oviductal ampulla and isthmus, and uterotubal junction and the uterus of the immature rabbit. They found that the uterus responded to 10 μg/ml PGE_2 by doubling the percent conversion of labeled nucleotides into cyclic AMP but synthesis was unaffected by a similar concentration of PGF_2. Isoproterenol incubated with immature rabbit uteri increased and oxytocin decreased adenyl cyclase activity in the immature rabbit uterus.

Easley _et_ _al_. (206) compared the effects of pentagastrin, a pentapeptide, on isolated uterine smooth muscle of virgin female rats with isolated guinea pig ileum and _in_ _situ_ uterine segments of the rat, dog and baboon. Pentagastrin stimulated uterine contractions _in_ _vitro_ and _in_ _situ_. The uterus was not as responsive to pentagastrin as was the ileum.

Wessels _et_ _al_. (207) review the role of microfilaments in cellular and developmental processes, and cytochalsin B as a tool to investigate these filaments. When the oviduct is removed from a five day old chick 36 hours after estradiol injection and placed in organ culture in the presence of cytochalsin B, all new gland formation ceases, glands already present regress by sinking back into the oviduct wall and the microfilament bundles are dispersed. The authors believe that this shows a positive correlation between integrity of the filaments and the morphogenetic process and that the critical role of calcium and high energy compounds in muscle contracting may be part of a primative, cytochalsin-sensitive system.

NERVE

Isolated nerves have been used to study the effects of compounds such as epileptogenic agents (208, 209), hallucinogenic agents (210), and narcotic analgesics (211) on nerve function. Ayala _et_ _al_. (208) used the crayfish muscle stretch receptor to study the effect of penicillin as an epileptogenic and stellate ganglion of the squid to study the effect of penicillin on an isolated synapse (209).

A desheathed cervical vagus nerve from a rabbit was used to demonstrate the effect of Δ^9 tetrahydrocannabinol on nonmyelinated nerve fibers (200). For monophasic electrical recording a sucrose gap apparatus was used. For diphasic recording the nerve was mounted in a glass capillary chamber in which five annular platinum electrodes for stimulation and recording were embedded. The compound action potential of the non-myelinated fiber was elicited each minute. The perfusion fluid contained Pluronic (a surfactant) in Lock solution, and 500 μM of Δ^9 tetrahydrocannabinol. The compound action potential fell by 7.5, 13.3 and 18.6% of its initial value after 15, 30 and 45 minutes of drug exposure. Threshold effects of Δ^9 tetrahydrocannabinol in this method were obtained at much higher doses than produce psychoactive effects in humans. However, it was demonstrated that Δ^9 tetrahydrocannabinol directly affects nerve fibers.

EPIDIDYMAL FAT PAD

The epididymal fat pad method has proven to be a sensitive and simple method for the bioassay of insulin. Addition of insulin to rat epididymal fat pads in vitro causes increases in glucose uptake, in CO_2 evolution with glucose as substrate in fat synthesis and incorporation of amino acids into protein (212). In this simple method epididymal fat pads from young rats are placed in an incubation medium containing Krebs-Henseleit or Krebs-Ringer buffer, test material, a substrate, and are equilibrated with 95% O_2-5% CO_2. Wide use of this method is made for studying glucose metabolism and lipolytic activity.

A glucose analog 6-deoxy-6-fluoroglucose was studied on both epididymal adipose tissue and rat diaphragm (213). 6-Deoxy-6-fluoroglucose was found to be an effective inhibitor of the oxidation of glucose. The stimulatory effect of insulin on glucose metabolism was blocked in fat tissue by N-ethyl maleimide, a sulfhydryl blocking agent, indicating a surface action on the cell rather than an intracellular effect (214). Ethylene diamine tetraacetate was found to potentiate insulin activity using the epididymal fat pad method (215). EDTA is known to chelate zinc and crystalline insulin contains 0.3 to 0.6% zinc (215).

Epinephrine and non-epinephrine were shown to stimulate production of nonesterified fatty acids and glycerol from rat adipose tissue when incubated in rat plasma (216, 217). The effect of eight amino acids upon glucose uptake of rat epididymal tissue showed significant inhibition of glucose uptake by arginine, glutamic acid, histidine, leucine, lysine and tyrosine (218). Colchicine, which produces fatty infiltration in mice and rats, did not show a direct stimulation of lipolysis in the in vitro fat pad test (219). Pyrazole is a potential inhibitor of alcohol dihydrogenase. Because of its possible effect on ethanol-induced fatty liver, pyrazole was studied using rat epididymal adipose tissue in vitro both on basal and theophylline stimulated lipolysis (220). The authors found that pyrazole does not reduce basal lipolysis but significantly decreases glycerol and free fatty acid release when lipolysis is stimulated (220).

The antilipolytic action of guanine derivatives on free fatty acid esterification was studied by measuring the incorporation of palmitate-1-C^{14} into the lipid fraction of rat epidydimal adipose tissue (221).

DISCUSSION

Isolated organs are useful and work well as a bioassay system for screening chemicals for specific organ directed activity. Three classical assay systems, the epididymal fat pad, the guinea pig ileum, and the rabbit aortic strip have been used for more than 20 years.

The epididymal fat pad is a simple and sensitive method for the bioassay of insulin (212). Wide use of this method is made for studying glucose metabolism and lipolytic activity. The guinea pig or rabbit ileum is used to study the effect of measurable amounts of drugs on intestinal activity. It has been widely used for antispasmodics, analgesics, and vasoconstrictors. Isolated rabbit aorta strips have been used successfully as a test system for studying the effects of various agents against epinephrine-induced contractions on vascular smooth muscle.

The isolated perfused liver appears to be a valuable system for studying the metabolic fate of materials and good correlation with the intact animal is frequently seen. This system has been used to study plasticizers, pesticides, and anaesthetic agents.

Piper and Vane (104) described a method using isolated guinea pig lungs which could provide a simple screening test for anti-inflammatory drugs.

Isolated segments of trachea have been used to study effects of cigarette smoke, aerosols, and air pollutants on mucus flow, muscle contractions, and ciliary activity. The results have been reproducible and correlate well with *in vivo* studies.

Everted segments of rat stomach and small intestine have given reproducible results. Lasagna (124) states that this method "not only allows the simultaneous study of drug metabolism and drug transport across gastric and intestinal mucosa, but can also provide information on the effect of drug metabolites on membrane transport of the drug." A striated muscle, the frog sartorius, has been useful for studying the effects of drugs on muscle contracture, metabolism structure and action at the neuromuscular junction. Caffeine and local anaesthetics have been studied using this method.

Isolated organs and tissues, including organ culture systems, have been very useful in getting at mechanisms of toxic action and providing an understanding of how a chemical exerts its toxic effect. In this role, such systems should become increasingly important. However, the use of isolated organs has serious limitations for studying toxicological effects, due in part to the modulating systems existing in the whole animal which can either increase or decrease an effect. The usefulness of these systems for toxicological screening also remains limited, primarily because such systems are most useful for screening large numbers of chemicals for a specific effect as opposed to screening a chemical for multiple biological effects. Additionally, in most cases, isolated organ systems use almost as many animals as an *in vitro* test would require. Consequently there is little saving in total animal use.

C. USE OF MAMMALIAN AND AVIAN CELL CULTURE SYSTEMS IN STUDIES ON CHEMICALS

INTRODUCTION

Cell Culture Methodology

Within the past 20 years, the techniques of culturing cells *in vitro* have been simplified so that their use is no longer restricted to highly specialized research projects. Techniques have become well defined and media and equipment are readily available to biochemists, microbiologists, pharmacologists, and other biological scientists. Perlman (222) has stated that there are many uses of animal cell cultures other than those related directly to virus and cancer research. These include (i) study of mechanisms of cytotoxicity and correlation of the cytotoxicity of drugs with other pharmacological attributes; (ii) study of the biogenesis of hormones and other "vital" products at the cellular level; (iii) determination of nutritional requirements of mammalian cells from "specialized" tissues or cells grown under unusual stresses; and (iv) study of host-parasite relationships at the cellular level. In fact, this list could now be expanded to include virtually any aspect of cell biology including aging, intermediary metabolism, cytogenetics, and cell cycle kinetics.

Smith *et al*. (223) reported that a statistical analysis on the correlation between tissue culture cytotoxicity and whole animal toxicity demonstrated a significant correlation for seven chemical classes of compounds. However, the correlations were not predictive for all compounds as markedly cytotoxic agents had relatively low acute animal toxicities and vice versa.

Although *in vitro* and *in vivo* correlations may not be absolute, cell culture techniques have created new approaches for assessment of chemical activities. Rapid bioassays for evaluation of primary and early events in carcinogenesis may be conducted in an environment free of host modifications and at the cellular level (224). The extensive testing, validation, and comparison with whole animal results can provide a useful additional method of screening for toxicity in spite of problems of relating cellular level results to their effects in the whole animal. It is of prime importance to note that potential toxicity may only be expressed as any significant change in the growth and function of treated cells when compared with untreated cultures.

HUMAN CELL CULTURE SYSTEMS

Human Carcinoma Cell Culture Systems

Human carcinoma cells have been used frequently in toxicological studies which include nucleic acid analyses, karotype analyses, as well as metabolic and cytological properties of cultures. HeLa cells (human cervical carcinoma), KB cells (Human nasopharynx carcinoma), and HEp 2 (human larynx carcinoma) are routinely used in such studies.

Kolodny (225) studied the inhibition of cell division by sodium chloroiridate on a variety of cells including HeLa cells. Confluent cultures exposed to 3×10^{-4} M sodium chloroiridate were pulsed at appropriate intervals with ^{3}H-leucine, ^{3}H-uridine, or ^{3}H-thymidine. Protein and nucleic acids were isolated following a 20-minute pulse at 37°C and counted. Results indicated that the compound inhibited synthesis of both DNA and RNA but not protein.

A potent carcinogen, 4-nitroquinoline 1-oxide (4NQO) was found to inactivate HeLa cells and Sendai Virus-carrier HeLa cultures (HeLa-HVJ) (226). Incubation periods in the presence of 10 µM 4NQO produced 10 to 100 fold inactivation of HeLa as measured by 30 to 60% inactivation of HeLa-HVJ cells.

The demonstration of an increased susceptibility of cells to the effects of ionizing radiation by combination with potentiating chemicals could be employed as both a biological radiation assay and as a practical application of radiation therapy. Hadacidin was found to potentiate the lethal action of X-radiation on a number of tumor cell lines, including HeLa, HEp 2, and KB cells (227). Cloning experiments were conducted in order to demonstrate additive cytotoxicity.

The cytotoxicity of Triton WR-1339 for HeLa cells was studied by Zimmerman et al. (228). The respiration rates, levels of Krebs cycle enzymes, and the morphology of mitochondria were altered by non-ionic detergent with this treatment.

Brookes and Duncan (229) conducted experiments on human embryo cells and compared their results with the effects produced on HeLa cells. The fates of benzo(a)pyrene (BP) and dimethylbenz(a)anthracene (DMBA) in cell culture was followed by use of tritiated hydrocarbons. The binding of BP to DNA, RNA, and protein was significantly less in HeLa cells than in human lung cells while the binding of DMBA was similar in both cases. Neither BP nor DMBA affected the plating efficiency over a range of 0.05 to 50 µM/ml of medium. The authors suggest that BP possesses transforming potential for human lung cells.

Toxicity studies using mycotoxins in HeLa cells have been reported (230). Crude extracts at levels of 5 to 20 µg/ml were observed to produce lysis of cells after 18-hour exposures. Monolayers of cells were trypsinized and counts of suspensions demonstrated cell cytotoxicity.

Chabbert and Vial (231) described a diffusion method for the study of the cytotoxicity of antitumor agents on HeLa and KB cells grown as monolayers (231). The monolayer is covered with agar medium and paper discs impregnated with the cytotoxic agent are deposited on the surface of the medium. After 16 hours, the agar medium is replaced by liquid medium and the culture is allowed to grow. A clear zone, indicative of toxicity, develops in the area where the toxic substance was deposited.

The action of 2,6 diaminopurine (DAP), a purine analog effective against transplantable tumors, was studied in KB cells. The compound was shown to stimulate glucose utilization and lactic acid porduction. Cytotoxicity of hadacidin and 2-deoxy-D-glucose was potentiated in KB cells by diaminopurine. The compound was administered in growth medium and cultures were observed for four to five days for cytopathology and for 48 hours for biochemical determinations.

An agar bioautographic system for analysis of cytotoxic agents was developed by Grady et al. (232). KB cell monolayers under agar are used as a support for developing paper chromatograms of actinomycin D, mitomycin D, streptovitacin, echinomycin, and fermentation liquors containing unidentified cytotoxic agents. After a 24-hour incubation, the agar and papergram were floated from the Petri dishes with Earle's salt solution. Zones of inhibition were observed directly, or by formalin fixation followed by Giemsa staining. The technique allows detection of multiple component cytotoxic activities and identification of components during purification.

The inhibitory effects of hadacid on KB cell cultures was studied by Neuman and Tytell (233). The growth-inhibitory substance which is active against human derived tumors was found to cause interference with purine metabolism.

The synthesis of DNA by HeLa cells as affected by dihydroxyphenylethylamine (Dopamine) was studied by Prasad and Kallmorgen (234). Several concentrations of dopamine (5-50 µg/ml) were added to culture media and radioautographic preparations using ^{3}H-thymidine were made as a function of time. The compound was found to reduce ^{3}H-thymidine incorporation into newly synthesized DNA and therefore inhibited growth of the cells.

The effect of methyl methanesulphonate (MMS) on replication of newly synthesized DNA in non-synchronized cultures of HEp 2 cells was studied by Coyle et al. (235). Cells were treated with MMS for one hour. The MMS was washed out and the cells were incubated in medium for various periods prior to addition of tritiated thymidine. MMS-treated HEp 2 cells were found to make DNA by semiconservative synthesis but that a portion of this new DNA is not itself replicated.

The effect of Dilantin on cell proliferation in cell cultures of human fibroblasts was studied by Shafer (236) and Houck et al. (237). Gingival fibroblasts and cutaneous fibroblasts were used respectively to determine if Dilantin did indeed stimulate cell proliferation in vitro which would be analogous to observations of decreased wound healing times observed in Dilantin-treated humans and animals. Dilantin at a concentration of 200 µg/ml was found to stimulate proliferation of gingival fibroblasts by cell couting procedures and a concentration of 2 µg/ml increased the rate of human diploid fibroblast proliferation. Tritiated proline analyses and total protein analyses did not demonstrate any effect on cellular collagen synthesis by Dilantin-treated fibroblasts. Cell doubling time, total protein, and collagen analyses could be employed to study chemical effects in other cell systems.

Human Lung Cell Systems

Human lung cell cultures have been employed as host systems for toxicity studies of chemicals, aerosols, and gases using lung explants, primary fetal cultures, and diploid fibroblastic cultures.

Leuchtenberger et al. (238) exposed human lung explants to puffs of fresh marijuana or tobacco smoke and performed chromosome and DNA analyses on treated cells. Feulgen microfluorometry and karyotype analyses showed that marijuana and tobacco smoke evoked abnormalities in DNA synthesis, mitosis, and growth which were observed early and persisted for prolonged periods after exposure. The use of in vitro systems for such studies provides rapid results and are more economical than when whole animals are employed.

Stockton et al. (239) developed a system in which human embryonic lung cells could be exposed to ozone in order to evaluate its effects on cellular proliferation and morphology. Cell culture monolayers, washed with protein-free BSS and inverted to insure removal of BSS and more direct contact with the gas, were exposed to several concentrations of ozone in air plus 5% CO_2. During long exposures, cultures were rinsed with BSs at frequent intervals to prevent cell damage due to dehydration. Ozone concentrations as low as 4 ppm appeared to have an inhibitory effect on cell proliferation possibly caused by alteration of cell membranes.

Barile and Hardegree (240) studied the effects of the oil-adjuvant emulsifying agent Arlacel A (AA) on a series of cell culture systems which included WI-38 human embryonic lung cell cultures. Cell culture monolayers were inoculated with AA by layering the monooleate onto the medium. After a three hour incubation, the cells were fed fresh medium. Cytotoxic effect (CTE) was scored at three days. Four of 19 lots of AA assayed by cell culture procedures were found to produce CTE. The 19 lots were also assayed by the Berlin test procedure to determine mouse toxicity. Observations of percentage of weight change and presence of peritoneal adhesions at sacrifice showed that 3 of 4 lots toxic by cell culture assay were toxic by the mouse assay. The findings showed that the cell culture assay is equally if not more sensitive than the routine mouse assay.

Aflatoxins, a mixture of toxic metabolites produced by Aspergillus flavus, the primary biological assay of which is conducted in Peking White Ducks, were studied in embryonic lung cells (L-132) by Legator and Withrow (241). The aflatoxins were dissolved in either propylene glycol or chloroform and added to culture bottles into which cell suspensions in medium were added. The chloroform, however, was removed prior to addition of cells. A 43-55% reduction in mitotic frequency was observed using slide preparations. The results of the study furnish a biological parameter of aflatoxin toxicity in which 0.01 μg of toxicant can be detected within 24 hours. The duckling and embryonated egg procedures detect levels of 0.25 μg within 10-21 days.

Carr and Legator (242) studied the effect of hexachlorophene on the metabolism of human embryonic lung cells. Through the use of pulsing studies employing tritiated thymidine, urine, and lysine during one cell generation, the authors observed a slight inhibitory effect on cell growth at a concentration of 1 µg/ml. DNA, RNA, and protein synthesis was not inhibited at hexachlorophene levels of 1 µg/ml but inhibition of the synthesis of these macromolecules was noted at concentrations of 5, 7.5, and 10 µg/ml. The adverse effects were thought to be associated with the uncoupling of oxidative phosphorylation.

Human Leukocyte Cell Systems

Human leukocytes cultured *in vitro* have been demonstrated to be extremely important research systems to evaluate the toxicological effects of drugs on cells of human origin. Procedures for culturing leukocytes are readily available and the method by which cells are obtained for study is not traumatic to donors. The indices used for leukocyte studies include chromosome analyses, phagocytic activity, DNA, RNA, and protein synthesis, and viability evaluations through use of counting procedures and vital dye exclusion observations.

The production of chromosomal aberrations following exposure of human lymphocytes to adriamycin (243), chlorpromazine, and meprobamate (244), ICRF 159 (1,2 bis dioxopiperazine 1-yl) propane (245), Busulfan (246), cyclamates (247), and acetyl salicyclic acid (248) have been reported. Routine orcein or Giemsa staining procedures were used to stain metaphase spreads prepared with colcemid, colchicine, or vinblastine sulfate following drug treatment.

Adriamycin was found to produce chromosome aberrations in leukocytes treated before or simultaneously with deoxyribose cytosine at levels of 0.05 to 0.25 µg/ml and 1 to 4 x 10^{-4} M/ml. Significant chromosome abnormalities were not observed when chlorpromazine (CPZ) was added to leukocyte cultures at concentrations of 5 x 10^{-5} to 2 x 10^{-6} M. However, blast transformation was inhibited by concentrations of 10^{-1} to 10^{-5} M. Meprobamate in concentrations from 10^{-3} to 10^{-4} M did not influence the rate of blast transformation as in the case with the CPZ-treated cultures.

The cytostatic agent ICRF 159 at concentrations of 1 to 10 µg/ml was found to block the entry of cultured human lymphocites into mitosis and stop dividing cells in prophase and early metaphase (G_2-M). Dose levels of less than 1 µg/ml of Busulfan produced no observable chromosomal alterations but doses of 1 to 20 µg/ml produced secondary constrictions and breaks. Cyclamate at a concentration of 200 µg/ml (15 g/75 kg) stimulated chromosome breakage in human leukocytes.

Human leukocyte cultures and leukocytes isolated from human volunteers were exposed to acetylsalicyclic acid in a dose range of 0.1 to 300 µg/ml. Leukocytes also were cultured from volunteers who had been treated with 2400 mg per day for 30 days. No significant chromosomal aberrations were observed in either system.

Savel *et al*. (249) and Lieberman *et al*. (250) studied the uptake of labeled thymidine to determine the effects of aflatoxin, proximate carcinogens, and alkylating agents on cultured human lymphocites. The addition of aflatoxin to phytohemagglutin-stimulated peripheral blood lymphocytes resulted in inhibition of tritiated thymidine uptake when compared to control cultures. In addition, aflatoxin suppressed thymidine uptake in lymphocyte cultures obtained from PPD and mumps reactive donors when the cultures were challenged with PPD and mumps antigens. In these assay systems, nitrogen mustard, methyl methanesulfonate, and ethyl methanesulfonate increased thymidine incorporation 7 to 8 fold above that noted in the controls. B-propiolactone and N-acetoxy-2-acetylaminofluorene stimulated incorporation of 4 to 9 times as much as controls. The demonstrations of increased thymidine incorporation suggests that DNA is being repaired following drug-induced breakdown. Whether the repair is functional is not known and studies at the molecular level must be conducted in order to determine functional repair and to identify cell damage and/or malignant transformation.

Lieberman (250) and MacKinney and Vyas (251) used dye exclusion tests to measure lymphocyte viability when the cells were exposed to carcinogens, alkylating agents and diphenylhydrantoin, respectively. Either trypan blue (0.1%) or erythrocin B (0.4%) in 0.15 M phosphate buffered saline was used to identify cytotoxic effects produced by the test compounds.

Hirshaut *et al*. (252) examined the effects of four clinically active antileukemic drugs in six long-term human leukocyte cultures. The drug concentration-time relationships (C x T) and dose response curves for 6-mercaptopurine, methotrexate, vincristine, and prednisolone were evaluated in cell lines RPMI 6410, AL-2, RPMI 8205, P_3J, RPMI 2217, and L 1210. All four drugs inhibited cell growth of long-term leukocyte cultures with the level of inhibition depending on the drug and the concentrations employed. The findings generally agreed with clinical observations in humans. The authors stated that *in vitro* procedures will not replace clinical trials but will, however, provide a tool in which C x T relationships might be predicted more rapidly.

Kvarstein and Stormorken (253) demonstrated that acetylsalicyclic acid, butazolidine, colchicine, hydrocortisone, chlorpromazine, and imipramine inhibited the uptake of polystyrene latex particles and oxygen consumption by human leucocytes. The drugs were added to cell suspensions, incubated for five minutes at 37^{o}C using varying concentrations and then exposed to 1.1 nm diameter polystyrene latex particles (PLx). The phagocytosis of PLx was quantitated and oxygen consumption of cultures during phagosytosis was measured using a Clark electrode. All the drugs tested inhibited both the uptake of PLx and oxygen consumption. The authors postulated that the phenomena may be caused by drug influence on lysosomal membranes or cell plasma membranes.

The effect of hycanthone (an antischistosomal drug) on phytohemagglutinin (PHA) stimulated human lymphocytes was studied by Sieber *et al*. (254). Five ml volumes of cells were exposed to hycanthone concentrations of 0.05 to 50 µg/ml. Labeled precursors, ^{3}H-thymidine, ^{14}C-uridine, and

^{14}C-leucine, were added to cell suspensions two hours before harvesting at 48 hours. The cells were washed, macromolecules harvested and radioactivity in DNA, RNA, and protein measured by liquid scintillation spectrometry. The morphology and mitotic indices of PHA stimulated leukocytes was also observed. Protein synthesis was not inhibited at any time interval, whereas both DNA and RNA synthesis were inhibited at various times with maximum inhibition at 30 hours. When hycanthone was added at the beginning of the 48 hour incubation period, the mitotic index (MI) decreased to 50% of the control. The MI was reduced to 0 at concentrations of 20 µg/ml or higher. At concentrations of 50 µg/ml, chromosomal analysis could not be made due to extensive damage. In addition to in vitro studies, the authors conducted embryotoxicity studies in pregnant mice. Hycanthone was administered on days 6-11 of gestation by the subcutaneous route. The drug did not produce teratogenic activity although body weights of fetuses were depressed. Doses of 25-50 µg/kg increased fetus resorption.

Human Erythrocyte Cell Systems

Although specific cell culturing techniques are not available for erythrocyte studies, techniques are available to detect physiological changes produced by exposure to toxic compounds. Cell deformability, measured by changes in filterability, has been used to determine the effects of prostaglandin E_2, epinephrine, and isoproterenol (255). Osmotic fragility and hemolysis (256 and 257) have been used to evaluate cellular damage produced by p-chloromercuribenzoic acid, lead and mercury. The inhibition of erythrocyte agglutination by homologous antisera has been used to study the physiological effects of phenothiazines and antihistamines (258). Test compounds were added to the antisera and slide agglutination tests were performed.

Human Liver Cell Systems

Human liver cell cultures have been used for in vitro hepatotoxicity studies involving metabolic and cytotoxic parameters. Savchuck et al. (259) used a cell culture system to investigate the action of arsenicals on living systems. Chang's human liver cells were exposed to sodium arsenite, sodium arsenate, arsenobenzene, 4-nitrophenyl arsonic acid, 3 nitro-4-hydroxyphenyl arsonic acid, and arsonelic acid. The growth response of mammalian cells was determined after a period of continuous exposure to the arsenicals using DNA content as an index of size of cell populations. The DNA assay used was a colorimetric test for deoxyribose using trichloroacetic acid hydrolysis, p-nitrophenylhydrazine, n-butyl acetate extraction, and NaOH colorimetric development. Deoxyribose content was detected at 560 mµ. The cells were observed to tolerate all the arsenic compounds tested except arsenobenzene. The value of this study was to present a DNA assay which is useful in estimating cell populations in cell culture.

Dujovne and Zimmerman (260) amd Zimmerman and Kendler (261) evaluated the effects of two phenothiazine compounds, chlorpromazine (CPZ) and promazine (PZ), as well as structurally related analogues on the enzyme content of Chang's human liver cells. Exposure of Chang cells to each of the phenothiazines led to leakage of lactate dehydrogenase, malate dehydrogenase, asparatate aminotransferase and glutamic oxalacetic transaminase. The demonstration of enzyme leakage from tissue culture cells of hepatic origin exposed to phenothiazines in vitro was taken as presumptive evidence that these drugs alter or damage the permeability of cell membranes. It should be noted, however, that this class of drugs exert potent antihistaminic and anticholinergic effects by blocking cell receptor sites.

NON-HUMAN CELL CULTURE SYSTEMS

Hamster Carcinoma Cell Systems

The transformation of Chinese hamster and Syrian hamster embryo cells in vitro has been reported by a number of investigators including DiPaolo et al. (262, 263, 264), Casto et al. (265), Huberman et al. (266), and Sivak and VanDuuren (267, 268, 269).

The research presented in a major paper by DiPaolo (264), describes the morphological, oncogenic and karyological characteristics of Syrian hamster embryo cells transformed in vitro by certain carcinogenic polycyclic hydrocarbons and continues to explore the possibilities and results presented in previous papers (262, 263). The in vitro results are correlated with other in vivo experimentation demonstrating the efficacy of the in vitro transformation assay of hamster embryo cells to elucidate and predict carcinogenic compounds. Carcinogenic hydrocarbons induced a quantitative transformation on cells as detected by the production of discrete colonies after plating. This transformation was not observed in studies using pyrene (noncarcinogen) or solvents alone. Transformation was defined as a criss-cross pattern of both light and dense clones producing altered colonies which were not observed in the controls. Altered and normal colonies were then developed into established cell lines which were analyzed in terms of their morphology, oncogenicity and karyology. Cells of the established lines derived from each transformed culture formed tumors when implanted subcutaneously into hamsters. No tumors were produced when control cells were reinoculated into irradiated animals. The authors conclude that since the colonies were transformed only with the carcinogens and developed cell lines which produced tumors when injected into hamsters while the control untransformed lines did not, that the in vitro model is reliable for studies of carcinogenesis and is relevant to in vivo cancers (264). It was noted that the frequency of appearance of altered clones was related to the known carcinogenic potency of the compounds tested and that the toxicity increase with the amount of the compound was also related to its potency as a carcinogen. At a constant carcinogen concentration, the number of altered clones increased with the number of cells exposed (263).

DiPaolo, Donovan, and Nelson have also demonstrated that an in vitro transformation in Syrian hamster embryo cells using polycyclic hydrocarbons can occur with no concomitant cytotoxicity (270).

The transformation of hamster cells that were seeded to form colonies was enhanced when the cells were treated with either a flavone or benz(a)anthracene before the addition of a potent carcinogen, benzo(a)pyrene or 3-methylcholanthrene. 7,8-Benzoflavone and benz(a)pyrene prevented the cytotoxicity by the carcinogens while 5,6-benzoflavone did not. The authors thus showed that it is possible to disassociate the transforming and toxic properties of benzo(a)pyrene and 3-methylcholanthrene.

Umeda and Iype describe an improved system of comparison for in vitro transformation rates based on the cytotoxicity which is produce by chemical carcinogens (271). Almost all chemical carcinogens tested have cytotoxic as well as transforming effects on cells. Usually the transformation rate and survival curves are plotted independently against the concentrations of the carcinogen used. This makes it difficult to compare the transformation rate at equitoxic levels of different carcinogens. The authors overcame this problem by plotting the transformation rate against the relative plating efficiency of the carcinogen-treated cells. They used this technique in presenting their own data on studies of the effects of pH variation in the culture medium of Syrian hamster embryo secondary cells plated over lethally X-irradiated rat embryo feeder cells. These cultures with pH values of 7.8 and 7.4, were then treated with 9,10-dimethyl-1,2-benzanthracene (DMBA). Seven to eight days after the carcinogen treatment they were fixed in methanol and stained with Giemsa. The authors plot the data generated by the conventional methods previously described and by their own. Their procedure graphically depicted the correlation between the cytotoxicity and transformation rate induced by DMBA, although the different metabolites of DMBA may differ in their relative capacities for transforamton and toxicity.

Syrian fetal hamster cells were exposed to transforming doses of certain polycyclic hydrocarbons (7,12-dimethyl benz(a)anthracene, 0.05 μg/mI; benzo(a)pyrene, 10 μg/ml; 3-hydroxy benzo(a)pyrene, 10 μg/ml; benz(a)-anthracene, 6 μg/ml) and analyzed to show the early changes in chromosome number and structure after treatment (272). Metaphases were found having abnormalities in chromosome number and structure within 24-74 hours after exposure.

Cocarcinogenesis in hamster embryo cells, a concept previously described with the Rauscher leukemia virus-infected rat embryo cell lines is explored by Casto et al. (265). Pretreatment of hamster cells in vitro with carcinogenic polycyclic hydrocarbons markedly enhanced the transformation of these cells by an oncogenic adenovirus (SA7). Eighteen hours prior to addition of the virus treatment with benzo(a)pyrene, 3-methylcholanthrene, 7,12-dimethyl benz(a)anthracene, dibenz(a,h)anthracene, or dibenz(a,c)anthracene enhanced transformation but was inhibited when the cells were treated five hours after the viral addition. Non-carcinogenic hydrocarbons did not stimulate SA7 transformation. Transformation was enhanced in direct proportion to chemical concentration up to a point. Higher concentrations resulted in either a decrease or complete inhibition of the viral transformation. The data indicated that the stimulation of viral transformation resulted from a direct effect of the chemicals on the cells which increased their sensitivity to the adenovirus transformation.

Casto and DiPaolo (273) produced a major state-of-the-art review of cocarcinogenesis in vitro (273). However, in this paper, X-ray enhancement of chemical transformation was included as well as viral enhancement.

Transformation of hamster embryo fibroblasts by benzo(a)pyrene and 7/12-dimethylbenz(a)anthracene has been reported (269). Cells of the transformed lines had altered karyotypes and the benzo(a)pyrene induced line was tumorigenic in vivo.

The effect of phorbol myristate acetate on RNA synthesis in benzo(a)-pyrene transformed hamster embryo lines along with 3T3 and SV40·3T3 mouse cultures was studied (268). While the chemical enhanced RNA synthesis in the stationary culture of 3T3 cells, it did not affect the virally transformed mouse cell line or the chemically transformed hamster embryo line.

Syrian hamster embryo cells were included along with C3H mouse prostate cells as the only two systems wherein chemical carcinogenesis had been firmly established in 1971 (274). The binding of labeled carcinogenic polycyclic hydrocarbons to the DNA, RNA and proteins of the transformable cells in culture was demonstrated and certain correlations to in vivo work were described.

Sivak and VanDuuren (275) utilized a mixed culture system in order to assess the tumor promoting activity of certain tobacco leaf extracts and cigarette smoke condensate (phorbol myristate acetate). This system was described in previous papers with mouse 3T3 and SV40-3T3 cell lines (268, 276, 269). Briefly, the growth of clones of either virally or chemically transformed cells when mixed with an excess of contact inhibited (untransformed) cells is enhanced on exposure to tobacco leaf extracts, phorbol esters from croton oil and other chemicals.

N-2-fluorenylacetamide (FAA) and its two metabolic derivatives, N-hydroxy-N-2-fluorenylacetamide and N-acetoxy-N-2-fluoroenylacetamide, were studied with Chinese hamster cells for toxicity and mutagenicity and with Syrian hamster cell lines for toxicity and transformation. The frequency of mutations as measured by the production of 8 azaguanine-resistant colonies in Chinese hamster cells was calculated per 10^5 survivors taking into account the number of cells at the time of treatment and the percent survivors. Cytotoxicity, in the Chinese hamster cells which were plated, treated with the acetone-dissolved compound, incubated 6-8 days, methanol-fixed and stained with Giemsa, was expressed as the percent of the number of colonies in the treated dishes to those in the untreated dishes. Cytotoxicity and transformation was performed in the Syrian hamster embryo cells. The colonies, after plating, were fixed, stained and scored for transformation and percent survivors 7-8 days later. Cytotoxicity was calculated the same as for Chinese hamster cells and percent transformation was determined as the percent of the number of transformed colonies found as compared to the total number of colonies scored. Cytotoxicity, mutagenicity and transformation frequency increased with the concentration and degree of reactivity of the derivative FAA.

The most commonly reported alterations in malignant cells are an increased aerobic glycolysis and an inhibition of respiration by glucose addition (277). Aerobic glycolysis of hamster embryonic cells (HE) transformed with 4-nitroquinoline-1-oxide and its derivative, 4-hydroxyaminoquinoline-1-oxide HCl increased. Respiration was inhibited by glucose addition in the presence of pyruvate.

An investigation was performed to elucidate the mode of incorporation and binding of the carcinogen, 4-nitroquinoline-1-oxide (4-NQO) to hamster embryo cell (HE) cultures (278). This carcinogen is incorporated within a short time after introduction to the growth medium and is bound to the macromolecules of the HE cells. This binding persisted for 72 hours after treatment. The rate of incorporation of N-4QO is directly proportional to its concentration and inversely proportional to cell number.

Rhim and Huebner depict an in vitro transformation assay of 12 major fractions of cigarette smoke condensate using mouse and hamster embryo cell lines (279). Transformation to numerous fractions correlated well with the corresponding development of tumors in vivo.

The flavone, 7,8-benzoflavone (7,8-BF), at concentrations 10 to 20 times greater than that of the hydrocarbons, almost completely inhibited the metabolism of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene to their water-soluble derivatives in hamster embryo cell cultures (280). The isomer 5,6-benzoflavone (5,6-BF) induced some aryl hydrocarbon hydroxylase activity (AHH) in the cells, while 7,8-BF inhibited normal enzyme activity as well as induction of AHH by benz(a)anthracene. 7,8-BF protected hamster embryo cells against cytotoxicity induced by 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene, and 3-methylcholanthrene while 5,6-BF gave a slight protection at high concentrations. This data was compared to the effects of 5,6-BF and 7,8-BF on DMBA-induced adrenal necrosis and lung tumorigenesis in vivo, and certain mechanisms of action and inhibition were proposed.

The transitions between the stationary, or confluent, state and the active, or cycling, state were examined for hamster cells in vitro (281). Values of a number of cell parameters (fraction of cells synthesizing DNA, rate of DNA synthesis, amount of DNA per cell, growth rate) indicated that the cells entered the stationary phase in G_1 and then doubled their DNA content without further division. While the sensitivity to sulfur mustard or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was not significantly different for active or confluent cells, the sensitivity to actinomycin D was greater for the confluent cells. The conclusion drawn is that the "stationary phase" of confluent hamster embryo cells does not correspond to the G_0 of hemopoietic stem cells, and that a state of "no cell cycle" is not by itself sufficient to give resistance to the drugs studied. Also, the post-treatment ability of the cells to repair the damage caused by sulfur mustard or BCNU was not enhanced by a state of "no cell cycle."

Diamond studied the metabolism of some polycyclic hydrocarbons in mammalian cell cultures to determine the relationship between carcinogen-induced cytotoxicity and metabolism of the carcinogen (282). The metabolism of 3,4-benzpyrene (BP) and 7,12-dimethylbenz(a)anthracene (DMBA) was studied in Syrian hamster, rat, and mouse embryo, mouse fibroblast, human fetal lung and kidney and HeLa cell cultures. Two extraction procedures were employed to measure the metabolism of the initiated hydrocarbons in the cell cultures. One measured the metabolism of the hydrocarbon to "alkali-extractable derivatives" and the other to "water-soluble derivatives." The kinetics of hydrocarbon metabolism indicate that there may be a sequential conversion of the parent compound first to alkali-extractable derivatives and then to water-soluble derivatives. Each of the cell cultures, which was sensitive to the growth-inhibitory effects BP or DMBA was able to metabolize the respective hydrocarbon to water-soluble derivatives. Little or no ability to metabolize the hydrocarbons was detected in cells resistant to the cytotoxic effects. Two resistant hamster cell lines were exceptional in that they did metabolize the hydrocarbons to water-soluble derivatives. However, no alkali-extractable derivatives were recovered from the medium of these cultures.

In order to investigate whether or not the development of carcinogen-induced cytotoxicity is due to selection of resistant cells or to the carcinogenic properties of dimethylbenz(a)anthracene (DMBA), Diamond et al. studied the carcinogen-binding capacity to DNA, RNA, and protein, and carcinogen-induced cytotoxicity by Syrian hamster embryo cell cultures at various passage levels (283). The results suggest that primary cultures include many sensitive cells with a high binding capacity for DMBA. With successive passages the sensitive and/or transformed cells (high binding) are lost and the population becomes characterized by resistant, low binding cells.

Kihlman et al. describe an experiment demonstrating the ability of caffeine to increase the frequencies of chromosomal aberrations produced by various physical and chemical agents including UV and x-irradiation, 4-NQO, alkylating agents, and mitomycin in root tips of the broad bean, vicia faba, and in cell cultures of the Chinese hamster (CL-1) (284). Caffeine post-treatments strongly potentiated the frequencies of chromosomal aberrations from U-V, mitomycin C, thio-TEPA, and 4-NQO while not affecting the aberration frequency of x-irradiation. The data also indicate a correlation between the effect of caffeine on the production of chromosomal aberration and on the loss of proliferative capacity of the cells. The authors hypothesize that the same molecular mechanisms may be responsible for both types of effect.

While normal hamster cells were either susceptible or resistant to the cytotoxic effects of dimethylnitrosamine (DMNA), transformed hamster cells were always resistant (285). Normal hamster cells remained susceptible to DMNA when tested four months after the increase in cellular lifespan which was induced by the DMNA; however, at a later time in culture there was a selection of resistant cells showing a growth advantage in both in vitro and in vivo systems. N-nitro-somethylurea (NMU), a related nitroso compound, was cytotoxic to normal cells but even more cytotoxic to the transformed hamster cells. The differential effect of these chemicals appears related to the difference in the growth rate of the normal and transformed cells. DMNA has to be converted enzymatically to its cytotoxic intermediate while NMU apparently is nonenzymatically converted to a cytotoxic intermediate. DMNA contrasting with benzo(a)-pyrene (BP) was cytotoxic to normal human cells but like BP was not cytotoxic to transformed human cells. The authors believe this difference indicated that the enzyme required to convert DMNA to its cytotoxic derivative is not identical with BP hydroxylase.

A comparison was made of the biological activities and chemical properties of chlorambucil and Trenimon, alkylating agents used to treat malignancies, on V78-1 Chinese hamster cells growing in vitro (286). The cells were grown in a culture chamber in a temperature-controlled box under a microscope fitted with a flash and camera for time-lapse photography. Pictures were taken at 1.5 minute intervals for one generation before the chemical was added to the medium and for two generations afterwards. The study was directed at measuring the division delay and loss of colony-forming ability of the cells. The qualitative response of the two compounds was similar in respect to division delay; however, the ratio of doses to get the same quantitative effect is 4,000 chlorambucil to 1 of Trenimon. A similar large difference was observed in the sensitivity of the cells to the two compounds concerning colony-forming ability. The large quantitative differences between their biological activities is a reflection of the relative ability of the two compounds to penetrate the cell under the conditions of the cell culture.

In order to ascertain the nuclear alterations in mammalian cells induced by L-canavanine (CANA), hamster tumor cells from polyoma virus (PV) were grown in an arginine-deficient medium, treated with 2.2 mM CANA, trypsinized, centrifuged, dehydrated in ethanol, embedded in Epon and sectioned. They were then stained with uranylacetate and examined with an electron microscope. After four hours, the cells were observed to have nuclear alterations characterized by the formation of irregular aggregates of the nucleoplasmic contents which were frequently attached to the nuclear membrane. It was postulated that the formation of canavanyl protein-DNA aggregate interferes with DNA replication.

Freeman et al. (287) studied the activation and isolation of hamster-specific C-type RNA viruses from tumors induced by hamster-embryo fibroblast cell cultures, which were transformed by 3-methylcholanthrene and cigarette-smoke condensate. Although these cell lines were negative for infectious virus before inoculation into animals, the hamster-specific C-type RNA viruses were isolated from tumors or from cell lines derived from the tumors. The authors conclude that since the infectious C-type viruses are not usually shown in hamster tissues of normal or tumor origin, the chemical treatment and the activation of the viruses are positively correlated.

Lossio (288) and Lossio and Wigler (289) exposed synchronized Chinese hamster cells to chemicals in order to elucidate the stage of the cell cycle in which cytotoxic effects occurred. The technique of cell synchronization was by reversal of colcemid inhibition. A mitotic index of 95-98% was obtained and an almost pure culture of cells in metaphase was plated at time zero. The lethal damage induced by the exposure of these cells to various concentrations of 5-fluoro-2'deoxyuridine (FUdR) was not restricted to cells exposed during the period of DNA synthesis (288). The colony survival fraction observed after treatment for one hour with 5×10^{-5} M FUdR was extremely low (0.0001-0.0003%) whether the drug was administered during early G, late G, early S or middle S phase. Since the survival of cells treated with FUdR during mitosis was significantly higher (0.62), it seems that the mitotic cells were less sensitive. Depending on how long after the cells were removed from the FUdR, the addition of 10^{-7} M thymidine or conditioned medium either reversed the lethal effects of the chemical or increased the survival rate of the cells.

Certain thiopyrimidines were studied for sytotoxic effect on a synchronized clone of Chinese hamster cells with a generation time of 16 hours (289). All four analogues tested induced cytotoxic effects, as measured by colony-forming ability, which increased with the concentration of the chemical and the length of the exposure time. Short periods of treatment (one hour) produced little effect at low concentration; however, they affected the survival of the cells differently when administered at different stages of the cell cycle. Two peaks of maximum sensitivity, one at late H_1 and the other at H_2, correspond to the peaks of maximum RNA synthesis in mammalian cells. Thus, the cytotoxic effects of thiopyrimidine analogues are probably related to their interference with RNA synthesis.

The effects of ouabain on baby hamster kidney (BHK) cells were studied including cell growth, macromolecular synthesis, ATP content, intracellular concentrations of sodium and potassium, and the cell membrane potential (290). For the growth experiments, the cells were trypsinized from dense cultures and seeded on Falcon tissue culture dishes. The density determined by counting in a hemocytometer and the results were the mean of two replicate plates. Ouabain inhibited growth, reduced the incorporation of ^{3}H-thymidine and ^{14}C-amino acids, promoted a loss of cell potassium and a gain of cell sodium, and reduced the membrane potential at a level of 4×10^{-4} M. The recovery from ouabain treatment was dependent on the concentration of the drug and the period of exposure.

Baby hamster kidney fibroblasts and a polyoma virus transformed variant (PyY) were grown as monolayer cultures and treated with tritium-labeled samples of phenanthrene, benz(a)anthracene, 7-methyl benz(a)anthracene, dibenz(a)anthracene (291). After 24 hours, DNA, RNA, and protein was isolated from these cells. Similar experiments were performed with the tritiated K-region epoxides, dihydrodiols, and phenols prepared from these polycyclic hydrocarbons. The four K-region epoxides were clearly more reactive to the constituents of both cell lines than the parent hydrocarbons, or the corresponding dihydrodiols and phenols. The reactivity of these epoxides within a biological system is discussed with particular reference to polycyclic hydrocarbon binding, cytotoxicity, and carcinogenesis. A correlation appears between the extent of hydrocarbon metabolism and the amount of binding to cellular macromolecules. This fact plus knowledge of in vitro microsomal systems indicate that reactive intermediates are formed from polycyclic hydrocarbons by the action of certain microsomal enzymes which oxidatively metabolize aromatic double bands.

Latner and Longstaff showed that a baby hamster kidney cell line (BHK 21), when maintained in the presence of crude histone preparations for three days, underwent morphological and behavioral transformations similar to transformations obtainable with viruses and mycoplasmas (292). Many of the cells were multinucleated giants with serrated margins, which show some loss of contact inhibition. A marked tendency toward centripetal aggregation and multilayering was also envinced.

Mayorca et al. describe a conditional state of transformed phenotype on BHK_{21} $Clone_{13}$ cells transformed by dimethylnitrosamine or nitrosomethylurea, and on a single "spontaneously" transformed clone (293). The transformed phenotype (clonal morphology and the ability to plate in soft agar) is exhibited when the cells are grown at 38.5°C, while the phenotype is normal when cultivation occurs at 32°C. The conditional state of these cells does not extend to their growth characteristics in that their plating efficiency at the two temperatures in liquid medium is similar. Conversion from the normal to the conditionally transformed phenotype and vice-versa is produced by shifting the temperature.

Kao and Puck examined the behavior of four known carcinogenic nitroso compounds to determine whether they were mutagenic and, if so, whether there was any pattern in their ability to produce single gene mutations and chromosomal aberrations which could be correlated with their carcinogenic activity (294). Actively-growing Chinese hamster ovary cell monolayers were treated with these water-soluble compounds and then analyzed for survival of colony formation, induction of chromatid breaks and exchanges, and production of auxotrophic mutations. All the compounds were effective in producing cell-killing, chromatid breaks and mutagenesis. There was a 50,000 fold difference in the mean lethal dose values (Do) as shown by single cell survival curves; however, all the compounds yielded constant values for their efficiencies of production of chromatid breaks and single gene mutagenesis, but not chromatid rearrangements, when these were expressed in terms of the Do values. N-nitrosomethylurea, N-nitrosomethylurethane, and N-methyl-N'-nitro-N-nitrosoguinadine all produced single-hit survival curves while N-nitrosodimethylamine produced a multiple-hit survival curve. This system may offer distinct advantages for studying the interrelationships between mutagenic and carcinogenic actions in mammalian cells.

Kao and Puck have demonstrated a methodology to quantitate mutagenesis of mammalian somatic cells by physical and chemical agents (295). Using Chinese hamster ovary cell cultures (CHO), the authors have developed a system to induce not only single gene mutations but also additional auxotrophic mutants. They have refined a method for quantitating the efficiency of single gene mutations to specific auxotrophies. Mutagenesis in the forward direction has been measured after treatment of the CHO cells with ethyl methane-sulfonate, N-methylN'-nitro-N-nitrosoguanidine, hydroxylamine, an acridine mustard (ICR-191), caffeine, and ultraviolet and x-irradiation. The single cell survival curves and the efficiency of chromatid breakage and rearrangement were measured for each chemical agent. Similar measurements were also performed with a water-soluble carcinogen N-nitrosomethylurea which was shown to be effective in producing auxotrophic, somatic mutations. The authors believe these results offer promise of illuminating the relationships between cell killing, chromosomal aberration, single gene mutations and carcinogenesis produced by the various agents. The methods elaborated can be used in the routine testing of drugs, food additives, and environmental pollutants for mutagenic action in mammalian cells *in vitro*.

The neoplastic transformation of hamster lung cells after their exposure to cigarette smoke condensate and the effects of tobacco tar on untransformed cells was performed *in vitro* (296). The hamster lung fibroblasts were transformed into malignant cells after three hours of exposure to crude cigarette tar dissolved in ethanol (10 or 100 µg/ml). Primary injuries to the cells included nuclear pyknosis, cell necrosis, and an enlarged, vacuolated cytoplasm. These were observed between 2 and 48 hours post-treatment. In one instance, giant cells were noticed at about 48 hours after treatment. Transformation manifested by random orientation of the cells (piling-up and criss-crossing) and continuous growth *in vitro* for over 300 days, occurred 100 days following treatment. The transformed cells cultured for 100 to 160 days produced tumors when transplanted into the cheek pouch of hamsters *in vivo*. Five of the nine animals which were inoculated with 100 µg/ml of the tar-treated cells (HT-100 strains) over 160 days *in vitro*, died from the tumors while the others, along with one implanted with cells of a different strain (HT-10), were sacrificed for histological confirmation. The tumors, histologically, were pleomorphic fibrosarcomas. Low doses (1×10^5 or less) of control cells did not produce tumors after 270 days in culture while higher doses of 10^7 control cells or more produced tumors when injected into the animals.

Mouse Cell Culture Systems

Mouse cells and tissues have been extensively cultured and used for *in vitro* toxicity studies.

The L-929 clone of mouse fibroblasts have been employed as an assay medium for a number of purposes including analyzing plastic and rubber toxicity (297, 298, 299, 300, 301), alcohol toxicity (302), and the toxicity of various drugs and compounds (303, 304, 240, 228, 305, 306, 307, 308, 309).

Investigators from the Drug Plastic Research and Toxicology Laboratory of the University of Texas analyzed the toxic effects of plastic and rubber materials on L-929 fibroblasts (297, 298, 299, 300). The toxicity of a rubber accelerator was analyzed by incorporating the rubber chemicals on dyed cell monolayers and by perfusing chemical solutions onto asynchronous cell cultures in a Sykes-Moore tissue culture chamber (297). A phase-contrast microscope was used to record the toxic effects on the cell cultures. A tissue culture technique was demonstrated for screening the toxicity of plastic materials via direct contact of the plastic with L-929 cell monolayers in Eagle's medium (298).

Prior to this methodology, the standard procedures used in medical practice was an *in vivo* implantation technique originated by Brewer and Bryant (310). This consists of implanting small strips of a plastic sample into the paravertebral muscle of rabbits. After a period of 3 to 7 days, the animals are sacrificed and the implant sites are examined by both microscopic and histopathological methods. The *in vitro* contact technique is less expensive and more sensitive than the *in vivo* implantation method. An adaptation of the above method was required for those plastic samples having low density or odd shapes (299). An agar diffusion method to determine not only plastic sample but also solid and liquid extract toxicity was designed. Replicating L cells and nonreplicating chick embryo cell monolayers were covered with an agar/calf serum overlay and evenly dispersed neutral red stain. Plastic samples were implanted on the culture plate which was inverted and incubated. After 24 hours, toxicity was indicated by clear, colorless zones of dead cells around the sample.

Growth inhibition studies were used to depict the changes in mammalian cell cultures caused by the plastic additive, triethylcitrate (300). Cell populations were determined using the methods of McIntire and Smith modified by Hori (311). Then, after a simple extraction procedures, nucleic acids (total purines and pyrimidines) were measured by their absorbance at 268 mμ. It was noted that the inhibitory action of triethylcitrate was independent of the inoculum sizes used.

Wallace L. Guess produced a general paper on the state-of-the-art of tissue testing of polymers in 1970 (301). The acute effects on tissues of pure polymers such as polyethylenes and polypropylenes, etc., and of compounded polymers and their additives, the plasticizers (dioctyl phthalate, citric acid esters), stabilizers (organometallic compounds), colorants, ultraviolet screening agents, and fillers are described. The primary test systems in use, the rabbit muscle implantation technique, the agar diffusion cell culture technique using either mouse "L" fibroblasts or chick embryo cells, are compared.

The relationship of *in vitro* and *in vivo* toxicity of a series of methyl- and halogen-substituted alcohols was examined with respect to their octanol-water partition coefficients, charge and steric parameters (302). A high correlation was found between the tissue culture toxicity and the hemolytic activity of the compounds. The product of the intrinsic toxicity (slope of the dose-response curve in tissue culture) and the inverse of the octanol-water partition coefficient for aliphatic alcohols had a uniform relationship to acute in vivo toxicity (LD_{50}-single dose required to kill 50% of the mice in 7 days). Other predicted relationships between in vitro and in vivo toxicity were also confirmed. The growth inhibitory response of the "L' cells was employed for the tissue culture assay. Comparative analyses of the results were carried out with the Free-Wilson (purely mathematical) and Hansch-Fujita (thermodynamic equilibrium) models. These supported the conclusion that the tissue culture system conforms closely to the equilibrium (time-independent) model of structure activity proposed by Higuchi and Davis.

L-929 fibroblast monolayers were inoculated with Arlacel-A (AA), an emulsifying agent for oil-adjuvant vaccines, in order to evaluate its toxicity (240). A characteristic cytotoxic effect consisting of intracytoplasmic refractile bodies was produced and this cell culture system was deemed a sensitive and reproducible assay for the evalution of AA toxicity.

Quinacrine, a drug used to treat malaria, produced nucleolar fragmentation in L-929 cells cultivated in monolayers. Fedorko and Hirsch (307) demonstrated this effect by the use of phase contrast and electron microscopy and hypothesized that the structural changes in the "L" cell nuclei are related to the known binding of quinacrine to DNA.

Two studies utilizing different methodologies to determine the effects of antibiotics on L-fibroblasts have been reported (309, 312). In the first (309), the survival response of cultured L-cell monolayers treated with bleomycin showed that the drug not only exerts a lethal effect but also induces resistance in the cells that are not killed. In the other (312), 24 different antibiotics were tested for their effects on the multiplying ability of "L" cells in suspension culture.

Growth inhibition studies with L-fibroblasts, using 16 local anaesthetics was compared with intravenous mouse toxicity, threshold tissue irritant concentration and threshold intracutaneous local anaesthetic activity (303). Both mouse L-929 cells and liver cell cultures were used. Toxicity was determined by measuring the growth inhibition caused by the drugs. Cell population counts were determined at the beginning and end of each experiment by the "total purine and pyrimidine" method of McIntire and Smith (311) with complete inhibition corroborated by microscopic examination. A significant logarithmic correlation was derived between the threshold irritant concentration and the in vitro cell toxicity only.

The scissions caused by 4-nitroquinoline 1-oxide of proteins liking DNA in cultured "L" fibroblasts and the subsequent rejoining of this DNA when removed from the 4 NQO have been reported. The "L" fibroblasts were cultured in a protein and lipid-free synthetic medium (DM-120). Cell growth was then estimated by counting cells following the simplified replicate tissue culture methods. Colony-forming ability after treatment was determined by plating (fixing (methanol), and staining (Giemsa) the cells). and counting the colonies formed (305, 306). A sucrose density gradient centrifugation and a viscometric technique which detects the DNase activity in the pronase were performed. Strand breakage increased with the increase in concentration of the carcinogen. However, both single and double strand breaks became completely repaired with a concommitant restoration of growth after a sufficient incubation time in a medium without the carcinogen ("recovery incubation").

A number of cell lines including mouse L-929 fibroblasts were treated with the nonionic detergent, Triton WR-1339, to ascertain its effect on cellular respiration and mitochondrial morphology (228). The inhibitory effect on cellular respiration was related directly to the cytotoxic response of the cells to the surfactant. L-929 cells were conditionally sensitive, while AV-3 and HeLa were markedly sensitive and primary rat and chick embryo cells were insensitive. The most striking characteristic noted in the treated cells was mitochondrial damage. The conditionally sensitive L-929 cells appeared to repair the induced damage following the removal of Triton WR-1339 from the media.

Horikawa *et al.* (313) investigated the dark reactivation of damage which was induced by ultraviolet light in mouse "L" cells, porcine kidney cells and mouse Ehrlich ascites tumor cells *in vitro* to determine the difference in sensitivities of these cell lines to x-rays and ultraviolet light and whether or not the reactivation processes after cellular damage by these two forms of irradiation are identical. The induction of pyrimidine dimers into the cellular DNA is the primary form of damage caused by ultraviolet irradiation. Reactivation mechanisms which split or excise these ultraviolet-induced dimers were measured by the colony-forming ability of the cell lines. There were no striking differences to x-irradiation among the three cell lines, but there were different sensitivities to UV light, porcine kidney cells being most sensitive and Ehrlich cells least sensitive.

The transformation *in vitro* of C3H mouse prostate cells is considered by some researchers (314, 315, 274) to be a reliable model for chemical carcinogenesis. Carcinogenic polycyclic hydrocarbons, though chemically inert, undergo extensive metabolism in experimental animals as analyses of bile, urine, feces, and liver homogenates have shown. Since metabolism is necessary for the malignant transformation of cells, it is important to follow the kinetics of metabolite production in cells under conditions where malignant transformation can be obtained. Mouse C3H prostate cells are one of the several *in vitro* system in which the malignant transformation by polycyclic hydrocarbons can be studied quantitatively (hamster embryo, rat embryo, AKR mouse) and so are ideal models for this type of assay. The metabolism of five polycyclic aromatic hydrocarbons to water-soluble and organic-soluble products was studied in C3H cells under conditions suitable for their malignant transformation (315). Prostate cell monolayers were grown in BME, and after a period of exposure (8-12 days) to a medium containing the hydrocarbon in question, were methanol-fixed and stained. Cytotoxicity was expressed as the percentage of the number of colonies in treated dishes over the number of colonies in control dishes. No correlation was found between the carcinogenicity of the compounds *in vivo* or *in vitro* and metabolism to water-soluble compounds in these cultures.

After a transformation assay, the technique of utilizing the formation of water-soluble products from polycyclic hydrocarbons as a measure of metabolism *in vitro* was performed to investigate the influence of microsomal triphosphopyridine nucleotide-dependent enzymes on the malignant transformation (314). Since metabolic activation of chemically inert carcinogens is considered to be essential for their biological activity, this work pursues the hypothesis that the metabolic activation of certain carcinogenic hydrocarbons is carried out by microsomal mixed-function oxidases and that epoxides are the carcinogenic substances.

Tritiated hydrocarbons were added to medium in which C3H monolayers were growing (274). After incubation, the cells were washed, harvested, and frozen at -80°. They were then fractionated into alcohol-soluble and insoluble portions, and the cellular constituents were isolated by a modification of the procedure of Diamond *et al*. (316). In this manner, the binding of carcinogenic polycyclic aromatic hydrocarbons to the DNA, RNA, and proteins of the transformable cells was demonstrated. Thus the observation that the carcinogenic hydrocarbons are firmly bound *in vivo* to the nucleic acids and proteins of mouse skin was confirmed and expanded. Also a good correlation was found between carcinogenic potency *in vivo* and transforming activity *in vitro*.

C3H mouse embryo cells were cultured and grown in a medium suffused with 4-nitroquinoline 1-oxide (4NQO) (317). At various passage levels, the cultured cells were implanted subcutaneously into C3H mice for assay of malignant transformation. The tumor latency period *in vivo* of the 4NQO treated cell lines was much longer than that of untreated controls; thus the chemical delayed the spontaneous malignant transformation *in vitro* of the mouse embryo cells.

Smoke from tobacco and/or marijuana was used to expose mouse kidney cells (318, 319), epithelioid cells of mouse lung (320, 321, 319), and peritoneal macrophages (322) in order to evaluate its toxic characteristics. Leuchtenberger and Leuchtenberger (318, 319) demonstrated that, while puffs of unfiltered cigarette smoke and its gas phase evoked rapid destruction of mouse kidney and lung monolayer cultures, cultures exposed to puffs of charcoal-filtered cigarette smoke did not undergo significant alteration. The fact that the gas phase alone contains factors affecting cell proliferation is corroborated by *in vivo* studies which showed that the incidence and spectrum of tumors in mice were increased after inhalation of both whole smoke and the gas phase alone.

These experiments used a Filtrona CMS-12 smoking machine. Lung cell cultures were prepared on coverslips and exposed to puffs of fresh cigarette tobacco smoke and tobacco mixed with marijuana smoke from a Filtrona CSM-12 smoking machine (321). Comparisons of effects on morphology, mitotic index, and DNA synthesis in epithelioid cells of lung explants were then made.

The effects of air pollutants, especially $NaNO_2$, on mouse lung, rat lung, and rabbit endothelium were described (320). The cells were grown in Rose multipurpose culture chambers and, after attachment, exposed to the culture medium containing the $NaNO_2$. All cell types tested in vitro exhibited partial but reversible inhibition in oxidative activity during treatment with $NaNO_2$.

Mouse peritoneal macrophage cell cultures and rabbit alveolar macrophage cells were exposed to whole cigarette smoke or to its vapor phase in a vertical Perspex chamber (322). A radiotracer assay technique for the determinations of ^{3}H-RNA and protein synthesis was employed. The derived data indicated that short-term exposure to cigarette smoke severely inhibits protein synthesis in macrophages. However, if the macrophages are exposed to low levels of cigarette smoke for a longer time, both protein and RNA synthesis increase markedly. These data support electron microscopic evidence of both active protein synthesis in vivo in human smoker's lungs, and the apparently contradictory fact of a severely reduced rate of protein synthesis in rabbit alveolar macrophages in vitro as reported by Yeager (323).

Mouse peritoneal macrophages were also used to examine the cytotoxic effects of silica, diamond dust, and carageenan (324). Phase-contrast and electron microscopy, histochemical techniques for lysosomal enzymes, and measurements of the release of lysosomal enzymes into the culture medium were the methods of analyses.

Frei and Oliver (325, 326, 327) studied the action of the carcinogen methylnitrosourea (MNUA) on primary mouse embryo cells in mass tissue culture. In vivo methods have shown that tissue differences in the methylation of DNA after a single carcinogenic dose of MNUA influence the transformation of mouse embryo cells. The results indicated that the chemical enhanced the malignant transformation of these embryo cells. After recovery from the primary injury caused by MNUA, the cells undergo a stepwise transformation in which increased plating efficiency appears first, and is followed by the appearance of morphologically transformed colonies which are invasive when implanted into irradiated hosts. It was also shown that in the early phases of the malignant transformation, MNUA prolongs the S phase of the cell cycle without killing cells (328). These experiments failed to resolve the question of whether the acceleration of biological transformation was due to the induction of heritable change in the cells or to the selection of malignant cells present in the initial cell population.

Rhim, Creasy, and Huebner described the altered cell foci found in mouse-embryo tissue cultures that had been previously infected with wild-type AKR (RNA tumor) viruses which were observed 9-14 days after treatment with the chemical carcinogen 3-methylcholanthrene (329). These foci from transformed cells consisted of randomly oriented, piled-up, spindle-shaped cells, the colonies of which when heavily stained with Giemsa were grossly visible and countable. These changes in morphology were not detected in uninfected cells treated with 3-methylcholanthrene or in untreated cells infected with virus under the same experimental procedures. This cocarcinogenic system is suggested as a rapid, quantitative test for the measurement of the oncogenic potential of certain carcinogens. It is possible that the infectious, but non-transforming RNA tumor viruses provides nascent oncogenic information which, after activation by 3-methylcholanthrene, serves as the specific genetic determinant of transformation.

Mouse embryo cells have been compared with other cell lines (269, 279). The toxic and transforming effects of phorbol esters, tobacco lead extracts, and cigarette smoke condensate fractions on several populations of mouse embryo cell cultures were compared with effects on 3T3 cells. An *in vitro* transformation assay of 12 major fractions of cigarette smoke condensate on rat and mouse cells infected with C-type RNA tumor viruses and on uninfected hamster cells has been conducted also. The combined effects of RNA tumor viruses and carcinogenic compounds in producing transformation of various cell lines is a highly sensitive and reproducible test system for qualitative *in vitro* work (330). The same condensate fractions producing statistically significant tumor-inducing activity are also most active in transforming cells.

Since 7,12-dimethylbenz(a)anthracene (DMBA) initiates skin tumors more effectively during diestrus than during estrus and so may be inhibited by estrogens, Nebert *et al*. studied the effect of the steroids, 17-β-estradiol and testosterone on aryl hydrocarbon hydroxylase, an enzyme system which could be a common pathway in metabolizing both polycyclic hydrocarbons and steroids (331). They showed that the hydroxylase system is induced by the polycyclic hydrocarbons DMBA and 3-methylcholanthrene in mice *in vivo* and noted that, in mouse fetal cell cultures, 17-β-estradiol in the growth medium prevents hydroxylase induction only at concentrations 6 to 60 times greater than the levels of the polycyclic hydrocarbon inducer.

3T3 mouse fibroblasts and Simian virus 40-.3T3) are mammalian cell lines popularly utilized alone and with other cell cultures to determine chemical toxicity *in vitro*. Sodium chloriridate produced irreversible inhibition of cell division of 3T3 and SV-40.3T3 cells *in vitro* at doses of 3×10^{-4} M (225). Although DNA synthesis was blocked, RNA and protein synthesis continued and resulted in cells of very large size. Ammonia decreased cell multiplication and altered morphology to a greater degree in the 3T3 line than in the transformed line and this occurred with minimal changes in the pH of the culturing medium (332). Sivak and VanDuuren (268, 276, 269, 275) studied the effects of carcinogens and tumor promoting agents on assay media consisting of separate and mixed populations of 3T3 and SV-40.3T3 mouse fibroblasts. A notable property of the 3T3 (untransformed) cell line is its sensitivity to density-dependent inhibition of cell division. Thus, in mixed cultures of both transformed and untransformed 3T3 cells, the untransformed cells inhibit the outgrowth of the SV-40.3T3. If non-toxic amounts of phorbol myristate acetate (0.01 to 1.0 μg/ml), an active tumor promotor in mouse skin, are added to the mixed culture, the density-dependent inhibition is reversed and the growth of clones grom the transformed lines is enhanced. This phenomenon is also true for a number of other tumor-promoting agents including tobacco leaf extracts, phorbol esters from croton oil and other chemicals (268, 275).

While it is generally considered that transformed populations are more resistant to the toxic effects of the carcinogenic hydrocarbons than the comparable untransformed cells, this is not always the case. Even after a 13-19 day recovery period after exposure to benzo(a)pyrene, the transformed 3T3 cells when cloned exhibited toxic effects for over 20 generations. These results indicated a permanent change in cell sensitivity had occurred, although certain properties characteristic of neoplastic cells in culture were not observed. This and other results suggest that tumor promotion in vivo does not involve a selection brought about by some general toxicity of the promoting agent. Also, the inhibition of growth in mass cultures as an index of toxicity is a relatively insensitive parameter when compared to cloning efficiency (269).

At subtoxic levels, the growth of the transformed clones is directly related to the dose of the tumor agent and the density of the contact inhibition overlay. Since the loss of contact inhibition is a neoplastic characteristic, the method provides a short-term reproducible in vitro technique to determine the ability of agents to promote tumorgenicity. This method eventually may supplant the usual time-consuming initiation promotion in vivo mouse skin assay especially when applied to isolated chemical compounds or purified chomical mixtures.

Specific murine tumor cell lines are often cultured for use in assays. Previous papers (305, 306) dealt with the scissions of protein linking DNA by 4-nitroquinoline 1-oxide (4NQP) in mouse "L" fibroblasts. It was demonstrated that 4NQP and the carcinogen 4-hydroxylaminoquinoline 1-oxide (4-HAQO) can induce single-strand breaks in the DNA of cultured Ehrlich ascites and tumor cells (333). The sedimentation behavior of DNA from normal and carcinogen-treated cells was analyzed by the alkaline sucrose gradient method and autoradiographs were prepared of unscheduled DNA synthesis in carcinogen-treated cells. As in the previous studies, when the cells are incubated after treatment, most of the DNA fragments can be rejoined.

Murine L-5178Y lymphoma cells were cultured as a medium to investigate the biochemical effects of acronycine (334) and barbiturates (335) on nucleic acid synthesis. Both L-5184 cells and IRC rat monocytic leukemia cells were grown in suspension culture and subjected to acronycine, an antineoplastic alkaloid, at concentrations of 0.5-12 μg/ml. The drug rapidly inhibits culture growth which agrees with the known action of acronycine against the L-5178Y tumor in vivo. DNA synthesis is inhibited at higher concentrations, but is not a prerequisite of the arrest of culture growth. The L-5178Y cells were much more sensitive to the actions of the drug than the IRC rat cells.

The inhibitory actions of barbiturates, especially pentobarbital, upon the kinetics of cell growth and upon the synthesis of nucleic acids and protein in L-5178Y lymphoma cells and P-815Y murine mastocytoma cells grown in suspension culture was explored (335). Pentobarbital inhibited the growth and synthesis of nucleic acids and protein in the P-815Y mastocytoma cells. The inhibition increased with an increase in the concentration of drug and was also time-dependent with a high level of drug. Similar results were obtained in lymphoma cells synchronized by sequential treatment with thymidine and deoxycytidine plus Colcemid.

A population of DDT-resistant cells was selected from mouse L-5178Y leukemic cells grown in suspension in a model G-25 Brunswick Gyrotory incubator-shaker at 37°C by chronic treatment with sublethal concentrations of DDT (336). These cells also exhibited resistance to other DDT pesticide analogues. One clone of cells, after passing through a continuous culture of 300 generations in the absence of DDT treatment, retained the resistant characteristics of the parent culture.

A Sarcoma-180 cell line (S-180) which was maintained in a monolayer culture for 12 years and was sensitive to 4,4'-diacetyl-diphenyl-urea-bis-guanylhydrazone (DDUG), was compared with a resistant subline with respect to cross resistance to methylglyoxal-bis-guanylhydrazone (CH_3G), 2-chloro-4', 4'-bis(2-imidazolin-2-Y^1)terephtholanilide (NSC 3828), and Vincristine (BCR) (337). These lines were also compared in terms of the nature and rate of cellular uptake of DDUG and its intracellular distribution and binding. The cellular uptake rate for L-1210 leukemia cells in vivo, a system showing resistance to DDUG, was unchanged in relation to systems having no resistance. No metabolic conversion of DDUG was observed in sensitive and resistant L-1210 cells or rat liver in vivo. This observation may be correct for S-180 cells also.

Chloramphenicol (50 µg/ml) inhibited the rate of protein synthesis in mouse myeloma cells grown in suspension culture by 50% (338). While there is a decrease in the amount of globulin synthesized, the rate of synthesis per cell is unchanged. The observed decrease is traced to the inhibition of cell proliferation caused by chloramphenicol.

Takayama and Ojima investigated the photosensitizing activity of carcinogenic and noncarcinogenic polycyclic hydrocarbons on a mouse tumor cell strain isolated from a mammary carcinoma (339). These cells were exposed to each of 8 polycyclic hydrocarbons and then illuminated with white light from a tungsten lamp. The carcinogenic hydrocarbons (benzo(a)pyrene, dimethylbenzacridine, methylcholanthrene, dimethylbenzanthracene, and benz(a)anthracene) were found to be much stronger in photosensitizing activity than the noncarcinogenic ones (anthracene, pyrene, and acridine). Among the former compounds, benzopyrene is the strongest and benzanthracene the weakest. A positive association between photodynamic activity and carcinogenicity was demonstrated.

The cell uptake of benzopyrene occurred in as fast as one second of incubation. When the cells were illuminated, lysosomal staining with neutral red became markedly reduced. This alteration in lysosomal staining took place prior to the occurrence of any other detectable abnormatlities. The photodynamic effect produced by the combined use of a photosensitizing substance and light is due primarily to the cell autolysis by the hydrolytic enzymes which were released from lysosomes as a result of photodynamic damage to their membranes.

Jackson made a study on the effects of polychlorinatted biphenyl compounds (PCB) on the growth and DNA synthesis of Krebs-2-murine ascites cells grown in vivo and in vitro (345). Agreement exists regarding growth and DNA synthesis inhibition of cells grown in vivo and in vitro.

Rat Cell Culture Systems

A number of in vitro studies utilizing rat embryo cells in culture have been reviewed (341, 279, 342, 228, 343, 344, 315, 345). Rhim and Huebner (343) treated monolayers from a continuous rat embryo cell line (S-1193b) derived from Fischer rats with various levels of polycyclic aromatic 1193b) derived from Fischer rats with various levels of polycyclic aromatic hydrocarbons (3-methylcholanthrene, 7,12-dimethylbenz(a)anthracene, and benzo(a)pyrene or dimethyl sulfoxide (control)). The cells treated only with the carcinogens underwent transformation in vitro. Newborn rats were then inoculated subcutaneously with freshly trypsinized cells in order to determine the transplantability of the transformed cells, and progressively growing transplantable tumors were, in fact, produced.

Gelboin, Kinoshita and Wiebel, in an important paper, explore the induction and role of microsomal hydroxylases in the toxicity and carcinogenesis of polycyclic hydrocarbons (346). Utilizing primary and secondary cultures of whole hamster, rat, and mouse embryos, 3T3, HeLa and a variety of other cell cultures, they demonstrate the mechanism for the induction of aryl hydrocarbon hydroxylase in vitro.

While the enzyme system of the liver generally converts polycyclic hydrocarbon to either weakly carcinogenic or noncarcinogenic compounds, it may also be involved in the activation of these compounds to either carcinogenic or toxic metabolites. Among the evidence for this dichotomy, the authors demonstrate that the toxicity of the polycyclic hydrocarbons to cells grown in culture is directly correlated with the presence of aryl hydrocarbon hydroxylase activity; that the inhibition of hydroxylase activity in cells by 7,8-benzoflavone is paralleled by an inhibition of the toxic effects of 7,12-dimethylbenz(a)anthracene (DMBA); and that 7,8-benzoflavone inhibits the enzyme in mouse skin homogenates and inhibits DMBA-induced skin tumorigenesis.

The benzine extraction of airborne particulate matter collected in Los Angeles yeilded carcinogenic material (374). The residue from this benzene extraction contains substances, some organic, which are soluble in methanol. The methanol extract and some of its component fractions were tested in certain rodent cell culture systems which were developed as assay methods for the screening of carcinogen compounds. Both a high-passage Fischer rat embryo cell line, and a low-passage cell line derived from NIH Swiss albino mouse embryos and infected with AKR leukemia virus were used. In both systems the methanol extract demonstrated cell transformation activity approaching that of 3-methylcholanthrene. The major inorganic component of the extract fractions, ammonium nitrate, showed no activity on the mouse cell system. Neither the neutral alone nor the recombined non-neutral fractions separately showed any activity either, although they were active together. The methanol extract combined with the conventional benzene extract showed much lower activity than either extract alone.

Rauscher C-type RNA murine leukemia virus infected rat embryo cells were the primary assay vehicle in transformation studies for Freeman et al.

(342, 344, 348), Rhim and Huebner (279, 349, 350), Hetrick and Kos (345) and Price et al. (351, 352). This system was developed to study the interrelated effects of murine leukemia viruses and exogenous chemical agents. The rationale for evaluating the C-type virus-infected embryo line as an oncogenic inducer is that these viruses possess all the characteristics necessary to implicate them as the basic determinants in a wide variety of leukemic and non-leukemic neoplasias.

Secondary rat embryo cultures were treated simultaneously with 0.1 µg diethylnitrosamine (DENA) and either CF-1 or Rauscher C-type RNA virus (342). Control cells were treated with either the chemical or virus alone. The virus-infected and DENA-treated cells alone remained basically unchanged. However, those treated with both became overgrown with randomly oriented spindle cells between the sixth and twelfth subcultures. Cultures treated with virus and DENA lost their normal and diploid condition and became aneuploid after later subcultures. This evidence suggests that the C-type RNA oncogene provides specific information for the transformation event. Rat cells in culture are ideal for in vitro transformation studies since they rarely, if ever, undergo spontaneous morphological transformation. The assumption that a malignant transformation has occurred must be corroborated by producing transplantable tumors. Special in vivo techniques for producing these in rats are required for a variety of reasons including inherent resistance in the rats.

Over 30 polycyclic hydrocarbons, azo dyes, aromatic amines and other chemicals were tested to see if in vitro transformation of high-passage rat embryo cell cultures infected with Rauscher leukemia virus (RLV) correlated with the known carcinogenic activity of the same compounds in vivo (344). The criteria for transformation included the development of macroscopic foci of spindle cells, lack of polar orientation and contact inhibition and the development of tumors when transplanted into newborn Fischer rats. Although certain exceptions were recorded, the in vitro results generally indicate that transformations were induced by known carcinogens but not by their noncarcinogenic analogues.

Freeman et al. also tested extracts of particulate matter from condensates of city air for their ability to transform rat or hamster cell cultures (348). Rat embryo cultures chronically infected with Rauscher leukemia virus (RLB) were transformed by benzpyrene or by extracts of city smog while uninfected rat embryo cultures were not. The smog extracts were 600 times more active than pure benzpyrene as transforming agents.

Hamster embryo cell cultures infected with hamster leukemia virus (HaLV) were equally as sensitive as the RLV infected rat cultures to the transforming effects of the smog; however, uninfected hamster cultures were also transformed, although tenfold higher doses of city air extract were required to do this.

The transformation of Rauscher leukemia-infected rat embryo cultures in the presence of foreign compounds was reported in two other cases (279, 345). The first is a work which was previously described using mouse embryos which also used RLV-infected rat embryos (F-119 line) and uninfected rat embryo cell lines (F-111). This was a transformation assay for possible environmental carcinogens, specifically on fractions of cigarette smoke condensates. The results correlated well with previously performed in vivo work in that a smoke fraction showing high tumorigenicity in vivo also had high transforming qualities in vitro.

Low levels of the antischistosomal drugs, hycanthone and lucanthone act as cotransforming agents in RLV-infected rat embryo cell cultures (F-1706 line), while neither the RLV infection or the chemical alone produced a transformation (345). Foci of the transformed cells appeared on the first subculture after chemical treatment and eventually became the predominant cell type. The transformed cells also exhibited the classic criteria of loss of contact inhibition, random oriented spindle cells, and tumorigenicity when implanted subcutaneously into newborn rats.

The transforming effect of benzo(a)pyrene (BP) was compared in three cell lines in vitro by Rhim et al. (349). A rat embryo cell line infected with Rauscher leukemia virus, a Swiss mouse line infected with AKR wild-type C-type RNA virus, and an uninfected hamster-embryo line when treated with BP were all transformed and produced tumors when reintroduced into homologous hosts. The infected mouse-cell cultures had transformed foci 9 days after BP treatment, while uninfected mouse and rat cells did not transform or cause tumors in vivo. The infected mouse-cell line was the most sensitive system for chemical transformation. Oncogenes of the RNA tumor virus genomes were derepressed by the action of BP which induced the transformation in the cells.

Rhim et al. continued their investigations into carcinogenesis by describing the malignant transformation which was induced in rat embryo cells infected with Rauscher leukemia virus by 7,12-dimethylbenz(a)anthracene (DMBA) (350). As in prior studies, no transformation occurred in cultures either treated with the chemical alone or infected with the virus alone. The foci of the transformed cells had much more rapid replication rates than the untransformed and untreated embryo cells and were randomly oriented and spindle-shaped. While the transformed cells produced local sarcomas when reintroduced subcutaneously into newborn rats, the infected or DMBA-treated untransformed cells did not. Cells derived from these tumors were reestablished into tissue culture and like the tumor tissue itself, contained group-specific complement-fixing antigens characteristic of the murine leukemia-sarcoma virus complex and the C-type RNA particles.

Price, Suk, and Freeman investigate the effect of the sequence of treatment of Type C RNA tumor viruses on Fischer rat embryo cell cultures as a determinant for chemical carcinogenesis (351). These cultures were treated with 3-methylcholanthrene before or after inoculation with Rauscher murine leukemia virus. Transformation was not observed in the untreated control cultures, cultures given virus or 3-methylcholanthrene alone, or cultures treated first with 3-methylcholanthrene followed by inoculation with the virus after removal of the chemical. Transformation was dependent on the presence of the Rauscher leukemia virus at the time of chemical treatment.

Price *et al*. studied the oncogenic potential of the four major cannabinoids found in marijuana (352). High passage Fischer rat embryo cells were inoculated with Rauscher leukemia virus and then treated with 1.0 µg or 0.01 µg per ml of each cannabinoid. The data indicate that the cannabinoids, with one exception, are inactive as transforming agents in this assay stem. The one exception, (-)-trans Δ^9-tetrahydrocannabinol, produced a transformation only after extended subculture (13 passages) of the cells. The activity of this cannabinol is weak in relation to that of 3-methylcholanthrene.

The antiviral and antitumor activity of hydroxyguanidine is described relative to its use as an antitumor drug (341). Hydroxyguanidine was examined for *in vitro* activity against the Moloney sarcoma virus, Rauscher pseudo type M-MSV (RLV) by assaying for its effect on focus forming units and/or *in vitro* cytotoxic effects (cell growth inhibition) in four tumor lines including L-1210 and L-51784 leukemia, Novikoff hepatoma and Walker 251 carcinosarcoma. The drug was also assayed for its activity *in vivo* against four experimental tumors. The drug exhibited definite antiviral and cytotoxic (antitumor) effects *in vitro* as well as antitumor activity *in vivo* and shows that other compounds combining the dual moieties of antiviral agents and antitumor drugs would possibly show potent chemotherapeutic activity.

Rat embryo cultures were employed, along with human cell lines, to depict the sensitivity of cells treated with the detergent Triton WR-1339 (228). Inhibition of the respiration rate of cells and the amount of mitochandrial damage was related to the cytotoxic response of the cells to the compound. While the respiration of certain sensitive cell lines (AV-3; HeLa) was markedly inhibited at low doses, the respiration of insensitive lines like primary rat and chick embryo cells was unaffected at much higher levels.

The metabolism of polycyclic aromatic hydrocarbons in cell cultures was studied in a number of cell lines under conditions for their malignant transformation. The primary line utilized was C3H mouse prostate cells; however, rat embryo and hamster embryo cultures were also used.

Other rat cell lines employed to explore *in vitro* cytotoxicity are rat heart (353, 354), rat fat cells (355), rat lung (320), rat thymus lymphocytes (356), rat pulmonary and peritoneal macrophages (357), rat liver (358, 359, 360, 361), and rat kidney (362, 363).

The interaction of carbon monoxide and hypoxia on the growth and contractile ability of cultured rat heart cells was described (353). Carbon monoxide sustained the contractile activity of the cells, reduced their growth, and affected cell toxicity as measured by nonviable cell count in a concentration-dependent manner when the oxygen tension was maintained at 20%. A simultaneous reduction in oxygen tension enhanced the inhibition of cellular growth by CO_2 and produced some reduction in the contractile rate. Inhibition of growth was much faster in cultures exposed to both reduced O_2 tension and CO_2 than in those exposed to reduced tension alone.

Lechat et al. studied the effects of the tricyclic antidepressant, imipramine, on cultured rat heart cells (354). After five minutes of contact at concentrations which were higher than 5 μg/ml, imipramine stopped the beating of cultured rat myocardial cells. This inhibitory effect on beating was suppressed in a K^+-free medium. After three days of contact, imipramine induced a vacuolization of both the myoblast (muscle-like) and fibroblast-like cells in a medium with or without K^+. Apparently this effect is nonspecific for cardiac cells as it was also induced by the drug on HeLa cells. Two different mechanisms to explain the effect of imipramine on the cultured heart cells are proposed. The first mechanism, which concerns the immediate effect of vacuolization, could be related to an effect on membrane permeability; the second, which occurs later and includes cessation of cell beating, could result from a general cytotoxic effect.

Desmethylimpiramine (DMI) blocks the lipolytic effect of norepinephrine, ACTH, theophylline, and dibutyryl cyclic AMP in isolated rat fat cells (355). Rat cells were prepared from epididymal fat pads. Various biochemical tests were then performed in which the antilipolytic activity of DMI was quantified and mechanisms for its effect were discussed.

Isolated cells harvested from trypsinized rat and mouse lungs were examined in tissue cultures to determine the effects of $NaNO_2$ (320). All the cell types studied showed a partial but reversible inhibition in oxidative activity during treatment with $NaNO_2$. Electron microscopy also revealed changes in the nuclear shape and mitochondrial ultrastructure during NO_2 treatment.

The lymphocytolytic activity in vitro of one of the nitrogen mustards, methylbis (beta-chloroethyl) amine, was investigated (356). The experiment was designed to study the influence of this chemical on aerobic glycolysis in a suspension of rat thymus lymphocytes. Incubation of the suspension was carried out in a Dubnoff Metabolic Shaking Incubator, and the protein was precipitated. The lactic acid in the supernatant was then determined and the results were expressed as the percent stimulation of aerobic glycolysis. MBA not only possesses lymphocytolytic activity but also stimulates the aerobic glycolysis of thymus cells.

In vitro cell culture methods were used to measure the toxicity of polypropylene using polyvinyl chloride with a known toxic additive (5% dibutyltin diacetate) as a positive control (357). The tests utilized measured the viability, adhesiveness, phagocytic activity and structure of rat pulmonary and peritoneal macrophages and the viability and mitotic activity of calf kidney and chick embryo fibroblasts cultured in the presence of extracts of the plastics in plasma. The tests were shown to be sensitive indicators of the presence of toxic materials. The in vivo effects were also studied by infusing two dogs with quantities of the polypropylene extract.

The malignant transformation of a diploid strain of rat liver parenchymal cells (RLC-10) treated with 3.3×10^{-6} M 4-nitroquinoline 1-oxide (4-NQP) in culture was explored (359). Rat liver cells were used because they transform spontaneously *in vitro* less often than do mouse or hamster cells. In each of five experiments the treated cells became malignant. They were backtransplanted after the treatment intraperitoneally into two suckling rats to confirm this malignancy. Continuous cinematography over a six-month period revealed no marked change in the static morphology of the treated cells but did show a loss of intercellular adhesiveness suggesting some change in the cell membrane. The untreated controls did not produce tumors on *in vivo* reimplantation. However, they did undergo spontaneous transformation by the seventeenth month.

A study was conducted to describe aryl hydrocarbon hydroxylase activity inducible in primary fetal liver cell cultures by various microsomal enzyme inducers (358). This experimental model in cell culture may be beneficial for comparing the effects of certain drugs, insecticides, and polycyclic hydrocarbons on cell oxidase activity. The maximum net rate at which the hydroxylase activity accumulates is approximately the same when phenobarbital, 3-methylcholanthrene, or benz(a)anthracene is in the growth medium at optimum concentrations. An additive effect is obtained when either phenobarbital or *p,p'*-DDT is present with a polycyclic hydrocarbon in the growth medium but not when the cells are treated with phenobarbital plus *p,p'*-DDT or with a combination of two polycyclic hydrocarbons.

Bendict, Gielen, and Nebert describe a study demonstrating polycyclic hydrocarbon-produced toxicity, transformation and chromosomal aberrations as a function of aryl hydrocarbon hydroxylase activity in cell cultures (361).

Phenobarbital prevents the cytotoxic effect of benzo(a)pyrene (BP) in fetal rat hepatocytes *in vitro*. BP is not toxic to HTC,A9 or HeLa cells in which the aryl hydrocarbon hydroxylase activity is either absent or very low. However, BP is cytotoxic to each of these lines when they are grown together with the liver cells in the presence of phenobarbital. While chromatid breaks are associated with polycyclic hydrocarbon-produced cytotoxicity, aneuploidy is more closely correlated with the malignant transformation of hamster secondary cultures. Benz(a)anthracene of α-naphtho flavone competitively inhibit the hydroxylation of other polycyclic hydrocarbons such as the carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) or BP. Also, the exposure of the fetal hamster secondary cells to excessive amounts of benz(a)anthracene prior to, during, and following treatment with DMBA prevents malignant cell transformation from occurring.

NLW cells derived from the liver of a newborn Wistar rat were transformed by aflatoxin B_1, a potent liver carcingoen, *in vitro* (360). The cells were cultivated for 161 days (12 subcultures) and then exposed to aflatoxin B_1 for 5 to 7 days at concentrations ranging from 10 to 0.01 ppm. They were then cultivated further in a maintenance medium. A delayed cytotoxic effect was observed for several weeks after exposure, especially at the two-week period, after which the surviving cells transformed morphologically.

After these cells were cultured for 87 days after aflatoxin exposure, they were transplanted into Wistar rats and produced recognizable fibrosarcoma. The results indicate that aflatoxin B_1 is a potent carcinogen *in vitro* and is able to produce malignancies *in vitro* at wide ranges of concentration.

Pelling *et al*. assessed a number of methods used in the safety testing of medical plastics (363). The toxicity of a variety of plastic medical devices (catheters, tracheostomy tubes, syringes, etc.), heart valves, and polyvinyl chloride (PVC) samples of known constitution were evaluated. The agar overlay tissue culture technique of Guess *et al*. (299) using primary rat-kidney cells and mouse L-929 cultures, implantation tests in female rabbit muscle and male rat subcutaneous tissue, examination of the systemic effects of administering extracts of plastics to rats and mice, and red-cell osmotic fragility tests were compared for the demonstration of toxic effects. PVC samples which contained known concentrations of an organotin compound were used as positive controls. While none of the test samples registered toxicities as high as those of the positive control plastics, the tissue culture technique was the most sensitive mothod used. Implantation of the rabbit sacrospinalis muscle for 7 days was the most sensitive of the two implant systems tested and the results showed a good correlation with the tissue culture. The systemic tests on plastic extracts failed to demonstrate toxicity, even with the positive control plastics.

Grasso, Gaydon and Hendy assessed lysosomal changes as an index of toxicity in cell cultures as a screen for the safety testing of medical plastics (362). Four samples of polyvinyl chloride (PVC) which contained 0, 0.17, 0.50, and 1.4% of dibutyltin diacetate but were otherwise identical were used to evaluate two tissue culture methods commonly used in the safety testing of plastics. In the agar overlay technique, primary neonatal rat kidney cells were grown in petri dishes, covered with 1% agar and stained with neutral red. The pieces of the test plastics were placed on the agar and the plates were examined after 24 hours. The area of neutral red loss under the plastic samples was the index of toxicity. In the other technique, primary neonatal rat kidney cells were maintained in a growth medium of serum which was previously used to extract dibutyltin diacetate from the PVC samples. Lysosomal acid-phosphotase activity was used in addition to the loss of neutral red to indicate cytotoxicity. The extent of cell necrosis was in direct proportion to the concentration of dibutyltin diacetate in the plastics for both methods. Nevertheless, the agar overlay method was the more sensitive in detecting low concentrations of the cytotoxic agent in the PVC samples. The histochemical method did show cytotoxic changes in the cells earlier than did the loss of neutral red but did not improve the sensitivity of the serum extract technique.

A rapid *in vitro* technique was developed and used in determining the effects of heavy metals on the cytotoxicity of thiosemicarbazone and other antitumor agents (364, 365). The method determined respiration-related cytotoxicity and was a modification of the Arai-Suzuki agar-dilution test system in which no serum is needed so that metal ion concentration can be rigidly controlled (366). Serial dilutions of potential antitumor agents were incubated in direct contact with freshly isolated tumor cells, normal cells or cells from tissue culture lines. The effect of the agent was measured by the decrease in dehydrogenase activity as a direct reflection of decreased respiration, with methylene blue as the indicator. Because of the short time necessary to run the test (<3 hours) no serum was required in the medium. The rat Walker 251 carcinosarcoma was the primary assay culture although other lines were also used including Jensen Sarcoma, Murphy-Sturm lymphosarcoma, carcinoma 755, and C3H mammary sarcoma (364). The test proved quite consistent statistically, and also elucidated the effects of copper and zinc ions on the cytotoxicity of thiosemicarbazone. The data indicated that a nontoxic level of copper ion (0.4 μg/ml) could activate thiosemicarbazone which by itself has little activity. This agrees with the *in vivo* observation that thiosemicarbazone is an effective antitumor agent in animals fed a diet high in copper but inactive when the diet is deficient in copper.

As was reported previously within the mouse section, IRC rat monocytic leukemia cultures were used in conjunction with L-51784 mouse lymphoma cells to test the effects of acronycine on nucleic acid synthesis and population growth. RNA syntheses was rapidly inhibited at concentrations of 0.5-12.0 μg/ml but culture growth is only arrested at much higher levels.

Rabbit Cell Culture Systems

Many studies have used primary rabbit alveolar macrophages to investigate the specific action of various pollutants on the lungs. Several reports deal with cigarette smoke as either a vapor or aqueous extract. Among its many actions on lung function, cigarette smoke interferes with the phagocytic activity of alveolar macrophages. Green (367) studied various reducing agents for their possible protective action on the cells against cigarette smoke. Freshly harvested alveolar macrophages were placed in tissue culture flasks with *Staphylococcus albus* bacteria and appropriate chemicals. Six ml of freshly drawn cigarette smoke was introduced by syringe and the cells were incubated for two hours after which the bacterial counts were made on the cultures, the counts serving as an index of macrophage function. It was observed that both glutathione and cysteine prevented the toxic effect of smoke in a dose-related response. This protective role of sulfhydryl agents suggests an oxidant action of cigarette smoke on these pulmonary cells. An effect on the cell membrane is suggested by the observation that coincident with the loss of phagocytic activity, cells exposed to cigarette smoke separated from the flask surface although they did not lose their ability to exclude vital dyes.

Green and Carolin studied the depressant effect of cigarette smoke on the *in vitro* antibacterial activity of rabbit alveolar macrophages (368). They developed an *in vitro* quantitative phagocytic system in which cigarette smoke was added to a mixture of rabbit alveolar macrophages and *Staphylococcus albus* in plastic tissue-culture flasks. The subsequent changes in the numbers of cultivable bacteria were determined by quantitative bacterial culture. The smoke had a marked depressant effect on the phagocytic activity of the alveolar macrophages. This inhibiting action varied quantitatively with the volume of smoke used, the type or brand of cigarette, and the kind of filtration (mechanical or aqueous) employed. The active component of the smoke was largely contained in the gaseous and filterable phase. Nicotine, acetaldehyde, formaldehyde, and cyanide did not, by themselves, affect the alveolar macrophages in doses comparable to their content in smoke. Cell viability, as measured by the ability to exclude vital dye, was not affected by the smoke.

Powell and Green (369) used aqueous extracts (by agitating smoke with water or Hanks' balanced salt solution) to investigate the enzymological basis for impairment of phagocytic function resulting from the exposure of alveolar cells to smoke. Enzymatic (by histochemical methods) and phagocytic (quantitative bacterial culture) determinations on the alveolar macrophages after two hours incubation with smoke extract were made. Supplementary experiments on the effect of smoke on cell-free enzyme systems were also performed. A concomitant loss of phagocytic competence and loss of enzyme activity after exposure to smoke extract point to a relationship between macrophage phagocytic ability and the activity of glyceraldehyde 3-phosphate dehydrogenase.

Again using an aqueous extract of cigarette smoke, Yeager (370) studied its effect on protein synthesis in *M. bobiv* induced alveolar macrophages. Uniformaly labeled L-leucine-^{14}C was used as the radioactive substrate and the appearance of radioactivity, measured in a Beckman liquid scintillation counter, in the washed trichloroacetic acid insoluble materials of alveolar cells is assumed to represent *de novo* protein synthesis. It was observed that the water soluble constituents of cigarette smoke depress protein synthesis in rabbit alveolar cells *in vitro*. This effect is dose dependent, partly reversible and greater than can be accounted for by the increase in acidity caused by the addition of a smoke solution to cell suspensions. It occurs at concentrations of smoke solution which do not affect cell viability or protein turnover. The major portion of this depressant activity is in the gas phase.

While Yeager had reported the reduced incorporation of L-leucine-^{14}C into protein, other workers presented electron microscopic evidence of increased protein synthesis in alveolar macrophages lavaged from human smoker's lungs.

Therefore, Holt and Keast (322) were prompted to reexamine protein synthesis in macrophages in response to a number of tobacco smoke regimes. Both mouse peritoneal and rabbit alveolar macrophages were investigated. It was found that short-term exposure to cigarette smoke (both vapor and aqueous extract) severely inhibits protein synthesis in macrophages while low levels of smoke for long periods result in marked increased synthesis of both protein and RNA. These results suggest that macrophages have the capacity to adapt to toxic changes in their environment.

Other than cigarette smoke, little is known about the effects of specific gases on alveolar macrophages, the prime defense mechanism of the alveolar areas. Hence, Weissbecker _et al_. developed a simple, rapid method for the determination of _in vitro_ alveolar macrophage viability after exposure to a variety of common air pollutants by the dye exclusion test (371). A hanging drop of rabbit alveolar cell suspension was exposed to gases in an air-tight chamber for one hour; an experimental model which attempts to reproduce conditions of the lower respiratory tract. The effect of the various gases on the macrophage viability is given in chart form. Some gases were found to interfere effectively with the defense provided by the macrophages.

Because of the suspected action of lung irritants on pulmonary defense mechanisms, studies were performed to assess the potential toxicity of particulate forms of vanadium oxides on rabbit alveolar macrophages in roller cultures (372). Cytotoxicity (using cell death as an end point) was determined to be directly related to solubility. Macrophage damage from vanadium oxides and its relation to urban air concentrations of this metal and acute pneuminities is discussed.

Allison used rabbit alveolar macrophages in parallel with primarily mouse peritoneal macrophages to determine the mechanism by which silica kills macrophages (324). The toxicity of silica particles stems from the fact that the particles are readily taken up by the macrophages and react readily with lysosomal membranes causing secondary lysosome formation and release of lytic enzymes. This study is an illustration of selective toxicity; the silica is selectively toxic to macrophages.

Kessel, Monaco and Marchisio designed an _in vitro_ study to test the specificity of the cytotoxic action of silica (373). In an attempt to elucidate the pathogenesis of silicosis, they inoculated monolayers of guinea pig peritoneal macrophages, cultures of rabbit and rat macrophages, human cell (macrophages, neutrophils, plasma cells, erythrocytes) and some primary tissue explants (skin, liver, heart, and muscle of chicks), along with the continuously cultivated monkey kidney and KB cell lines with media containing sterile, powdered silica, hemalite and carbon. Cytotoxicity was measured by the ability of the cell cultures to metabolize. The supernatant culture fluids were analyzed for lactic acid produced per unit of time by both chemical and enzymatic means. In general, the data indicate that an appropriate surface is necessary for the characteristic cytotoxicity of silica, and that this toxicity is specific for the macrophage.

Monkey Cell Culture Systems

Toxicities of 15 analogous compounds (374), related to either sterigmatocystin or aflatoxin B_1, were evaluated on primary monkey (_Cercopithecus aethiops_) kidney epithelial cells grown on glass coverslips in roller tubes. They were exposed for 24 hours to the various compounds which were dissolved in Hank's balanced salt solution to a final concentration of 2 mg/liter. The effects of these compounds on nucleolar morphology, mitosis, and incorporation of ^{3}H-thymidine and ^{3}H-uridine were studied. Structurally, these compounds were divided into three groups manifesting distinct toxic patterns which indicated a correlation between toxicity and structure. Apparently an unsaturated $\Delta^{1,2}$-furobenzofuran system and the position of methoxy and hydroxy groups on the xanthone ring of the sterigmatocystin analogues affected the cytotoxicity in these cell cultures.

Cultures of a monkey kidney (*Cercopithecus aethiops*) cell line (R-1 CA; hypodiploid, 57-59 chromosomes), containing reduced proportions of anisonucleolinar nuclei, were treated for 8, 24 and 48 hours with various doses of thioacetamide (TAA, a water-soluble carcinogen) and hydroxylamine (HA, a mutagen) in the media (375). It was tentatively assumed that the nucleoli may be a critical site for the carcinogenic impact. Thus, these cultures were observed for nucleolar and mitotic alterations. These alterations were observed 24 and 48 hours after TAA administration. A good correlation was found between the developmental patterns of anisonucleolinosis and abnormal mitoses in the TAA treated cells. HA failed to induce anisonucleolinosis but induced smaller nucleoli with no changes in their ability to incorporate ^{3}H-uridine.

Monkey kidney cell cultures were also used to study the effect of bleomycin (an antibiotic) on cell survival. From these results, discussed in the mouse section, some principles of bleomycin chemotherapy are outlined (309).

Chicken Embryo Cell Culture Systems

Chick embryos have been used extensively for biological studies, both whole embryos and monolayer cell cultures prepared from their tissues. Guess *et al*. (297, 299) have shown that chicken embryo cell monolayers overlaid with nutrient agar and stained with a vital dye can be used successfully to screen the toxicity of various chemicals and/or materials. In both of these studies replicating mouse cells were used in parallel with the nonreplicating chick embryo cells. More details of these studies can be found in the section dealing with mouse cell cultures.

In 1969, Wilson and Stinnett (376) studied the effect of two agricultural chemicals, malathion and malaoxon, on two primary cell systems, chick embryo heart cells and pectoral muscle cells, using a respiration chamber constructed specifically to measure oxygen consumption of monolayers of cells growing on coverslips. Cells were exposed to insecticide containing media for periods up to 60 minutes and then counted. Both 3×10^{-4} M malathion and malaoxon are strongly toxic to the growth of pectoral and heart muscle cell cultures and malathion is more toxic than malaoxon. An acute dose of malathion, 3×10^{-4} M inhibits cell respiration; however, incubation of the cells in 3×10^{-4} M malaoxon does not. The evidence presented suggests that malathion inhibits the energy metabolism of chick embryo cells and that the conversion of its P→S group to the P→O group of malaoxon results in a disappearance of the inhibition.

This paper also describes a respiration chamber suitable for the measurement of respiration of monolayers containing 200,000 or more cells.

It consists of a polarographic system connected to a gas analyzer and strip chart recorder. Such a device could be very useful for the assessment of toxicity of potential respiratory poisons.

Wilson, Stinnett, et al. (377) studied malathion and malaoxon on respiration of chick embryo pectoral muscle cultures using the previously mentioned respiration chamber. Malathion and malaoxon inhibited growth (cell number, protein, DNA) of the cells. Low-protein phosphate-buffered medium with pesticide was more toxic than high-protein, carbon dioxide-buffered medium. Growth was also inhibited by parathion and paraoxon. Determinations of cholinesterase activities and isoenzymes of cell cultures and respiration of tissue homogenates, cells and mitochondria are included in this report. The observations presented give a detailed account of inhibition of pesticides of acetylcholinesterase in cells in culture. This study confirms the prievious study's finding that the inhibition of respiration may play a role in the toxicity of malathion to cultured cells.

For an evaluation of germicidal efficiencies and toxicities of a group of antibiotics intended for clinical application, Salle and Amesur (378) used an embryonic chick heart tissue fragment-saline suspension. Portions of the tissue suspension, bacterial suspension (Micrococcus pyogenes var. albus), and antibiotic dilution were combined in a tube and agitated in a 37°C water bath. After two washings the fragments were embedded in plasma in Carrel flasks and allowed to grow. A toxicity index for the antibiotics, a ratio of the highest dilution to prevent growth of tissue fragments to the highest dilution to kill test bacteria under test conditions, is obtained.

Dorsal root ganglia from chick embryos cultured by the hanging-drop method on collagen-coated cover glass in media containing different amounts of mercury compounds were studied by Kasuya to show the relationship between the structure of mercurial compounds and their toxicity on nerve tissue in culture (379). The adverse appearance and/or inhibition of migration and outgrowth of the cells were observed. From the results, it was postulated that the binding of mercury compounds by their hydrophobic and hydrophilic moieties with specific binding sites on membranes is a major factor in their toxic action. The author further suggests that by application of tissue culture methods, it may be possible to investigate the neurotoxicity of mercury compounds more accurately without considering such factors of absorption and distribution.

Racz and Marks (380), utilizing the intact chick embryo for the bulk of their investigation, made supplementary use of monolayer chick embryo liver cells to demonstrate the presence of active DDC and Ox-DDC, porphyria-inducing drugs, in cell culture media 24 hours after incubation.

Using chicken erythrocytes incubated with N^{15}-glycine as a test system, Abbott, Jr., and Gindin (381) compared the effects on heme synthesis of a series of benzimidazole derivatives having nitro or chloro groups instead of a methyl in the five position. Similar studies with 5-hydroxybenzimidazole are also included. This investigation was prompted by an earlier supposition that benzimidazole derivatives inhibit an important metabolic reaction fundamental to more than one biosynthetic process since they were found to inhibit virus duplication and prevent the incorporation of N^{15} from N^{15}-glycine into heme by chicken erythrocytes during *in vitro* incubation.

MISCELLANEOUS CELL CULTURE SYSTEMS

Rat Kangaroo

Palmer *et al*. (382) used an aneuploid cell line from the rat kangaroo (*Potorous tridactylis apicalis*) to determine whether the persistent presence of DDT or its major metabolites produced chromosomal abnormalities in an *in vitro* system. The cytogenetic effects of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD, *o,p'*-DDD, *p,p'*-DDE, *o,p'*-DDE, and *p,p'*-DDA were investigated by exposing various concentrations of the chemical, diluted in media, to the monolayers for 24 hours and then examining the cells for CPE, mitotic index and chromosome damage. It was found that the isomeric configuration of the compounds is related to the production of exchange figures; the *p,p'* isomers produced exchange figures and the *o,p'* isomers failed to produce exchange figures even though cells were observed with multiple chromatid breaks. These types of damage are probably of great mutagenic significance since they are the cells most likely to survive and carry an alteration of the genetic material.

Guinea Pig

Parazzi *et al*. (383) investigated the site of cellular damage induced by silica particles *in vitro* to guinea pig peritoneal macrophages. Tridymite (50 μ - 150 μ) and coal dusts (0.5 μ - 5 μ) were suspended in Hank's media immediately before adidtion to the cell cultures and remained in contact with the cells for various intervals up to seven hours. The early phases of phagocytosis were followed by measuring the release of some cell enzymes (LDH, ribonuclease, APH, SDH, GDH) into the culture media and by observing the integrity of the cell membrane using fluorochromatic methodology. The evidence presented suggests that when silica is added to macrophage cultures *in vitro*, it interacts with the external cellular membrane and that the first cellular lesion occurs at this site. Further damage might then be inflicted by the liberation of lysosomal enzymes. Coarse particles (50 μ to 100 μ) which could not be phagocytized by the macrophages showed that mere contact of the external cell membrane with the surface of the silica particles was sufficient to damage the membrane which was evidenced in a very short time by the loss of fluorochromasia and some escape of cytoplasmic enzymes. The coating of the particles with 20% guinea pig serum resulted in a delayed release of enzymes.

Pig

4-Nitroquinoline N-oxide (4-NQO), a potent carcinogen for mice and rats, has been found to be mutagenic as well as cytotoxic. Horikawa *et al*.

(384) investigated the 4-NQO-induced damage in cultured mammalian cells by comparing the response in three cell lines: mouse L cells, porcine kidney stable (PS) cells, and Ehrlich ascites tumor cells. The cells were suspended in 5 ml of culture media containing different concentrations of 4-NQO for various intervals and were then plated in chemical-free media and observed for colony forming ability. Toxicity was determined by directly plating cells in media containing various levels of 4-NQO. For the analysis of 4-NQO incorporation, cells were grown for various intervals in media containing ^{3}H-4-NQO and the radioactivity was determined by a liquid scintillation counter. It was concluded that PS cells are more resistant to 4-NQO than the other two cell lines and mouse L cells are the most sensitive to 4-NQO. In a previous paper the authors showed that Ehrlich cells were more resistant to ultraviolet light than the other two cell lines and PS cells were the most sensitive to UV. These results seem to indicate that the effect of UV on cells differs from that of 4-NQO. However, there are no differences in the incorporation rate of ^{3}H-4-NQO into cells among the three cell lines. The authors also suspect that the differences in ability among the three cell lines to reduce the compound 4-NQO to a more carcinogenic but less cytotoxic intermediate, 4-HAQO caused differences in sensitivity to 4-NQO. The activity of the reduction pathway in the target cells, which yields 4-HAQO, is thought to be essential for its carcinogenic mechanism as well as its cytotoxicity.

DISCUSSION

Cell cultures, particularly those of highly differentiated cell types, are important tools for physiological, pharmacological, and toxicological studies. Difficulties observed in many studies, however, reveal problems which have and might ultimately be encountered if in vitro systems are to be employed for the primary toxicological screening of pesticides and other pharmacological agents. Cell cultures consist of single cell types, in the case of established cell lines, or several cell types in the case of primary cultures derived from a whole organ, tissue, or embryo. Hence their study will not directly detect events which depend upon the integrative biological mechanisms of the animal. Two aspects of the problem caused by working in vitro must be borne in mind. In some instances metabolic activation must occur in order for a compound to become toxic.

Should a test cell culture system not include cells with this metabolic capability, a potentially toxic chemical may appear to be innocuous as a result of in vitro testing.

The other side of the coin concerns detoxification. The intact organism has a detoxification capability which may exceed that of an in vitro system.

Specific procedural problems which might affect the sensitivity and results of in vitro cell culture toxicity testing are as follows:

(1) Cell cultures may contain latent or occult viral agents which may produce false positive toxic effects or, in

carcinogenic studies, produce an unexpected cocarcinogenic effect.

(2) The test sytems may be contaminated with Mycoplasma which perhaps will produce cell degeneration due to nutrient depletion or cell infection or profound alteration in cell metabolism.

(3) Great care must be used in the selection of cell culture media and its constitutents, particularly the animal source of serum to be used, the supplier, and even the batch. These factors, as well as the presence or absence of antibiotics, must be controlled because they may adversely influence cell metabolism or they may form interfering complexes with the test compound.

(4) Transformation of cells by chemicals is not an absolute indication of the tumorigenic activity of the same chemicals in animal hosts.

(5) When immunologically competent cells are used for transformation studies, specific care must be used to select compatible allotypic cells which will not be affected by graft-host responses, or will not transform spontaneously.

(6) Specific toxic effects may not be manifested in early cell passages and a sufficient number of serial transfers must be conducted to allow observation of these responses.

The overall degree of correlation between in vitro and in vivo studies indicates that in vitro toxicity testing of chemicals, drugs, and physical agents is scientifically practical within specific limitations. In vitro methods, which allow a more careful control of agent concentrations and cell exposure times than is possible in animal systems, are simple enough to permit a large number of samples to be evaluated in a short period of time. Other advantages which might be realized by using in vitro systems are:

(1) When studies concern expensive or rare compounds, microgram quantities can be tested, whereas larger quantities are needed for bioassay in animals.

(2) The specific dose delivered can be closely controlled since the use of cell cultures circumvents physiological and metabolic effects which occur within the whole animal.

(3) The relative cost per compound tested in a large scale screening program has been estimated to be $1500 to $2000, a cost considerably less than long-term animal studies.

(4) In vitro toxicity testing procedures can be used to characterize concentration-time relationships which make it possible to estimate dose-time schedules for in vivo or clinical trials.

D. USE OF BACTERIA, FUNGI, PROTOZOA, AND PLANT CELLS IN STUDIES ON CHEMICALS

INTRODUCTION

In attempting to develop alternatives to long-term mammalian toxicity testing, the use of nonmammalian systems must be considered. The use of single cell organisms, which are easily cultivated in large numbers has an inherent advantage. Results can be obtained in a short span of time, and very large numbers of individuals monitored.

It is felt that extrapolation of information obtained in one cellular system to another more complex multicellular system is valid if a careful review of the affected subcellular systems is conducted. All cells have structural and metabolic properties in common; all cells contain DNA, are enclosed by semipermeable membranes, possess electron transport systems.

In reviewing the literature, a search was made for reports on the use of plant cells, protozoa, fungi, and bacteria to assess the toxicity of any chemical. Some of these reports were quite specific in detailing toxic effects at the subcellular and metabolic levels--others were concerned only with a plus-minus indication of toxicity. Correlations attempted by the authors between their cellular data and that from mammalian systems have been emphasized.

BACTERIA

The use of bacteria as indicators of compound toxicity goes back to the early studies on antimicrobial agents, and later antibiotic studies, where the bacteria themselves were the end point of desired knowledge. In recent years, however, more emphasis has been placed on the use of bacteria as indicators of drug effects in higher organisms and ultimately in humans. As the following discussions show, in some cases these correlations can be made, but in other cases they cannot.

Growth Inhibition:

Cells are exposed to the chemical under test either in broth or on agar plates, with observations on change in turbidity or zones of inhibition as the end points. These are methods widely used in assaying antibiotics and antimicrobials, and yield only a plus-minus qualification of toxicity, and no information on the biological mechanism of toxicity is obtained. *Escherichia coli* (175, 385, and 386) was tested in this manner against promazine and chloropromazine, 2,4-dinitrophenol, and various nitrosoguanidines. In one of these studies (386) in which strains of *E. coli* resistant to 1-methyl-3-nitro-1-nitrosoguanidine (NG) are used, it was found that these are resistant to a variety of "radiomimetic" compounds. In addition, one of the mutants seems to be specifically resistant to the precise chemical configuration of NG. The authors question if such specific events occur in mammalian cells, and could they account for resistance of tumors to radiomimetic agents.

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In a study of the toxicity of a toxin produced by *Alternaria mali* (230), it was found that some gram-positive cocci and some fungi were inhibited by the compound but *Bacillus sp*. and some gram-negative bacteria were unaffected. Mice and human cells in tissue cultures were also tested against the toxin, and the authors conclude that "the range of organisms--mice, human cells in tissue culture, fungi, and bacteria--adversely affected by the crude extract indicates that this isolate of *Alternaria mali* produces a broad spectrum of toxicity."

Some studies have yielded specific information on the biological mechanism of toxicity. *E. coli* was treated with ethionine (387) and the effect on cell viability observed. Cell extracts show that ethionine inhibits specific enzymes leading to protein synthesis, and it was stated that "ethionine is toxic to a broad spectrum of biological systems . . . from microbial growth inhibition to the genesis of a variety of tissue aberrations in mammals."

In a study (388) to determine the toxicity of a series of tetrazolium salts against seven bacterial species (gram-positive and gram-negative), it was determined that the ditetra salts were more toxic than the monotetra salts and that the growth inhibition is probably due to interference with cellular enzyme systems.

A herbicide, paraquat (1,1'dimethyl'4,4'bipyridilium dichloride), was assayed for toxicity against *E. coli* (389). It was found in this case that the compound was only bacteriostatic, reversibly inhibiting the synthesis of DNA, RNA, mRNA, and protein through interference with energy metabolism.

A group of food additives, all lipophilic acids, was tested against *Bacillus subtilis*, *E. coli*, and *B. pumilis* (390). The resulting growth inhibition was found to be due to the cell's inability to take up amino acids and phosphate groups.

A group of *Salmonella* mutants were used to study the toxicity of B-propiolactone (391). It was found that the chemical acts directly upon cell DNA. *Salmonella* were also used to assay the toxicity of a group of unsaturated carbonyl sugars produced during the irradiation of sugar solutions (392). These compounds inhibited the growth of these bacteria through irreversible reactions such as addition of sulphydryl groups and substituted amines to cellular constituents. Schubert and Sanders also determined that these compounds inhibit glycolysis and respiration in tumor cells, inhibit the growth of ascites cells and produce chromosomal breakage *in vitro*.

Clostridium perfringens has been used to study the inhibitory effects of a series of alcohols (393). The inhibitory effect increased with the molecule chain length, and it was also noted that lecithinase production by the cells was a more sensitive indicator of alcohol toxicity than was cell growth. Specific DNA synthesis was inhibited in a large group of bacterial species when treated with 2,3-dihydroxmethylquinoxaline-1,4-di-N-oxide (394). This compound has also been found to have activity in protozoa and viruses.

The studies discussed above used observations on the growth and/or viability of the treated cells as an indicator of toxicity. In a unique study (395), the ability of treated bacteria (*B. megaterium*) to convert to viable protoplasts was used as the toxicity indicator. Magnesium chloride and a series of cationic and anionic surface active agents were tested with this system.

Potentiators:

A great deal of interest has arisen in the effect of "potentiators" on the toxicity of compounds in test systems. In a study on the effect of UV irradiation on *E. coli* B (396) chloroquine was found to reduce the survival of irradiated cells, and appears to inhibit the dark repair mechanism. Hadaciden (N-formyl hydroxyamino acetate) was found to potentiate the lethal effects of ionizing radiation in *E. coli* (397).

In a study to select possible potentiators (398), 36 pairs of chemotherapeutic agents were assayed against *E. coli*. Wheeler, Schobel and Skipper state that the mode of action of chemotherapeutic agents appears to be the same in bacterial and mammalian systems. However, Guthrie *et al*. (399), reporting on "collateral sensitivity" to chemotherapeutic agents, found that the effects of a pair of antimetabolites on bacteria cannot predict the effect in another biological system. This group used mutants of *E. coli* and *B. subtilis* which were resistant to purine antagonists. They were exposed to the original drug plus another under test, using agar diffusion, and observed for inhibition of growth.

A more specific assay for toxicity is the determination of effect on the DNA of the treated organisms. A group of nitrofuran derivatives was used to treat *E. coli* B and B/R cells (400) followed by analysis of the lengths of the resulting DNA. It was found that the active DNA damaging compounds are reduction products of nitrofurans and that a direct correlation exists between the number of DNA breaks a compound produced in *E. coli* and the degree of carcinogenicity in mammals.

In recent years, the use of bacteria as indicators of chemical mutagenicity has gained importance. Ames has stated that since mutagens alter DNA, and the DNA of all organisms has the same structure and contains the same nucleotides, any organism may be used as an indicator system for mutagens (401). Bacteria offer many advantages over more complex material. It is possible to treat and screen a large number of cells in a short time. They have short generation times, small genomes, are haploid, and can be grown on defined media. Bacteria may be used to detect forward, reverse, and suppressor mutations. Both mutagenic and inactivating DNA alterations can be detected.

Great care must be used, however, in transposing the interpretation of data obtained in bacteria to what occurs in higher life forms. Bacteria lack some of the protective mechanisms of mammalian cells. Metabolic alterations of the chemical under test are different in bacteria and in mammalian cells; however, the development of the host-mediated assay and of the microsome activation preparations has overcome part of this difficulty. The uptake and transport of the compound may differ. Correlations are being made of data obtained in bacteria with data obtained in mammalian systems and it is expected that the dichotomy will be resolved.

Studies with mutagens in bacteria have been done mainly with the Salmonella tester strains of Ames (402, 403, 404, 405, 406). Ames and his coworkers have developed Salmonella histidine mutants which detect base-pair substitutions and frame shift mutations, through reversion to prototrophy. They are rendered highly sensitive to most mutagens through loss of a lipopolysaccharide coat and through deletion of a gene in the excision repair system. The chemical is spotted on an agar layer lacking histidine, containing the Salmonella and microsome preparation. Mutated colonies are the only ones able to grow and are directly counted. Ames has found that a wide variety of chemical carcinogens are detected as mutagens using this system. These carcinogens include polycyclic hydrocarbons, aromatic amines, nitroquinolines, aflatoxins, fluorenes, and can be detected at nanogram levels.

Hartmann et al. (407) in earlier work has used Salmonella mutants for rapid spot tests for mutagens. They found that hycanthone was mutagenic in this system, while miracil D, a closely related compound, was not. A mutant strain of E. coli which was deficient in DNA polymerase was growth inhibited by some well documented carcinogens, including nitrosomethyl- and ethyl-urea, the comparable urethans, MMS, EMS, hydroxyurethan, nitrosofluorene, and hydroxylaminofluorene. Dichlorovos and Captan, ingredients of several pesticides, were found to be mutagenic to an auxotrophic mutant of E. coli (408). A large number of pesticides were not mutagenic in this system.

A review article by Stoltz et al. (409) includes the mutagen screen system as one of the three offering promise in this area.

The development of microbial assay systems for mutagen detection is progressing, as can be seen, to a high level of predictability for mammalian systems. The use of these assays in combination with others in higher organisms can yield a great deal of information useful to those concerned with human safety.

Antitumor Compound Screens:

A group of papers published in 1958 outlines the rationale for the use of microorganisms in an ongoing screen of antitumor agents (410, 411, 412). The collaborative studies included the use of 16 microbial systems (bacterial, fungal, and protozoan) and human cell cultures of normal and neoplastic origin. The organisms for the multiple bioassay system were selected in order to base the in vitro screening procedure on a variety of diverse metabolic pathways. In the work reported by Foley, McCarthy and coworkers (412), the results obtained from microbial bioassay of 200 selected compounds are reported. In general, the organisms were grown in appropriate broth containing the test compound, and the concentration which allowed only half-maximal growth determined.

The microorganisms used included *Streptococcus faecalis*, *Excherichia coli*, five *Lactobacillus species*, *Leuconostoc citrovorum*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces carlsbergensis*, *Tetrahymena pyriformis*, *Glaucoma scintillans*, and *Colpidium campylum*.

Little direct correlation was found between the results obtained in the different microbiological systems. No single bioassay system detected all of the known active compounds. While *T. pyriformis* gave 82% identification only 39% were picked out by *Candida albicans*. Certain combinations of three or four of the bioassay systems can be selected which give comparable positive percentages as the whole group. The authors make the point that microbiological assay systems can be readily devised by choice of proper strain and medium to detect compounds exhibiting antimetabolite activity against any specific essential metabolite.

The limitations of any *in vitro* screen are (1) host metabolic-activation of a compound with no corresponding activation by the test system; (2) occurrence of indirect action; (3) inability of *in vitro* systems to handle as large a dosage as can be obtained in the *in vivo* situation; (4) instability of compounds during the duration of an *in vitro* test.

In an accompanying paper by Foley, Eagle and coworkers (410), the results of the previous study are compared with data obtained with experimental tumors *in vivo*. They found that mammalian cell cultures appear to be superior to microbiological systems in assessing the effectiveness of antitumor agents.

In a report by Schabel (411), another type of antitumor agent screening program utilizing bacteria is described.

In this screen, a combination of drug-resistant and drug-sensitive strains of *Escherichia coli* or *Streptococcus faecalis* are used to distinguish new agents with modes of action differing from known agents. Inhibition of the parent drug A-sensitive line with failure to inhibit the drug A-resistant line of the same organism suggest that compound X has a mode of action similar to that of compound A. These assays are done as spot tests, with a filter disk containing the test compound placed on a lawn of the suitable organism, and zones of inhibition observed. It is also possible to study the biochemical mode of action of known anticancer agents with this system.

Although these methodologies were not specifically designed to assay for toxicity in whole animals per se, they could be readily adapted to become useful tools for toxicity testing. A great deal of information on many different groups of compounds has been amassed in anticancer agent screens, and should be tapped for the more general purpose of *in vitro* toxicity assessment.

FUNGI

Fungi and mammalian cells possess some common metabolic pathways and enzyme systems, but it cannot be assumed that the entrance of a compound into a cell or the action of modifiers or "activators" on the molecule is the same in both types of cells. It has been found that certain unusual side effects of drugs in mammals can be predicted through the use of yeast, but it is not known if such inhibitors function in higher eucaryotes as in the lower (413). In addition, differences in structure, degree of specialization and environmental modification preclude any close correlation. On the other hand, where effects of chemicals are found to be upon structures or metabolic functions common to both cells, or where provision is made for "activation" of a compound, then such correlations can be usefully made. This is certainly true in the case of mutagens where the toxic action is on chromosomal material, as yeast cells and other fungi, being eucaryotes, have a closer relationship to mammals than bacteria, which are prokaryotic.

Growth Inhibition:

The direct toxicity of a chemical on the organism can be assessed through observations on growth and/or survival, or through analysis of specific disruptions in functions of the cell.

Using observations on growth, nitroquinolines and nitropyridines were assayed for toxic effects against S. cerevisiae (414, 415), and it was noted that there existed a correlation between the degree of inhibition of yeast by a compound, its degree of carcinogenicity in mammals, and its mutagenicity in lower organisms. Epstein and St. Pierre (44) point out the dynamic association found with this group of compounds: mutagenic in yeast <=>, carcinogenic in rodents <=>, photodynamic activity in protozoa <=>, phage induction.

In contrast to the above correlation, when a varied group of cholesterol synthesis inhibitors were studied, no correlation was found between the ability of a compound to inhibit cholesterol synthesis in rat liver homogenates, and to inhibit the growth of yeast cells (413, 416). In one of these studies (416), several species of yeast were used, including Hansenula, Candida, Geotrichum, and Saccharomyces.

Inhibition of growth, sporulation and morphological changes are produced in Aspergillus, Penicillium, Neurospora, Cladosporum, Mucor and Rhizopus species during exposure to a mycotoxin, rubratoxin. The work reported by Reiss (417) indicated that a toxin level of 10 μg/ml was sufficient to produce toxicity in some of the test species.

Genetic Damage in Saccharomyces:

The structural organization of chromosomal material in yeasts is comparable to that in mammalian cells. Forward and reverse mutations and reciprocal and non-reciprocal mitotic recombination events are easily studied.

In a study by Epstein and St. Pierre (414), respiratory deficient mutants of Saccharomyces cerevisiae were produced with nitroquinolines and detected on agar plates containing triphenyltetrazolium chloride. It was found that those compounds which were toxic to the cells were mutagenic. In a study (418) of the toxic effects of nitrous acid and alkylating nitrosamides on S. cerevisiae, it was found that the frequency of induced reverse mutations were dependent on the temperature of treatment.

When nitroquinolines, nitropyridines and their derivatives were studied (415), using the spot plate technique and DNA repair deficient Saccharomyces as the test organism, a correlation was found between yeast growth inhibition (due to lethal mutations) and previously reported carcinogenicity in mammals. This type of assay has been proposed as a screening method for carcinogens.

In a modified spot test system by Fink and Lowenstein (419) the effect of test conditions was shown. When EMS, NG, and DES were added to cells on minimal agar, no increase revertants was seen, but when cells were treated on complete media and then replicated to minimal, an increased reversion frequency was evident.

Mitotic recombinations are induced by base-pair substitution frameshift mutagens and carcinogens, and apparently occur at higher frequencies than do "mutations." In three papers (420, 421, 422), the induction of non-reciprocal mitotic gene conversion in Saccharomyces was studied with a group of pesticides and herbicides and their N-nitrosated derivatives (Benzthiazuron, Propoxur, and Carbaryl). A strain of Saccharomyces which requires both adenine and tryptophan is used, and the cells, after treatment, are plated on media containing only one of these compounds. Gene conversion creates cells which no longer require both compounds.

The non-nitrosated compounds had no influence on conversion frequencies, but the nitrosated derivatives displayed marked convertogenic activity. N-nitroso compounds display a wide range of biological activity (acute and chronic toxicities, carcinogenicity, and teratogenicity). The authors point out the possibility of conversion of the inactive pesticide in the human gut to these highly active forms. The same group also studied the genetic effects of a group of 32 herbicides in the same system. Only two of these herbicides showed activity (Diquat and 2,4-D).

In a study by Vogel, Fahreg and Ohe (420) a group of triazenes was tested in four genetic test systems: mitotic gene conversion in Saccharomyces; host-mediated assay using mouse and Saccharomyces; genetic abnormalities in Drosopyila; and chromosome aberrations in cultured human leucocytes. The triazenes have been described as "indirect mutagens," requiring metabolic activation for effectiveness. The three compounds tested were PDT (1-phenyl-3,3-dimethyltriazene), PyDT (1-(pyridyl-3)-3,3-dimethyltriazene), and PyNDT (1-(pyridyl-3-N-oxide)-3,3-dimethyltriazene).

In Saccharomyces, both PDT and PyDT produced convertants. The same was true in the host-mediated assay, although PyDT showed more activity than PDT, the reverse of the results in vitro. PyNDT was not active in either system. In Drosophila, recessive lethals were produced by PDT and PyNDT, with PDT being more active. Chromosome aberrations in human leucocytes were produced at very low levels by all three compounds. The authors conclude that for the detection of "indirect" mutagens, the host-mediated assay and *Drosophila* tests are clearly superior.

Another effect of mitotic recombination, reciprocal crossing over, is the basis of a test system using *Saccharomyces*. Mitotic crossing over has been demonstrated in a wide range of organisms including *Aspergillus*, *Drosophila*, soybean, and mouse cells. In the *Saccharomyces* test system the strain D5 carries two recessive alleles of the gene ade-2 (colored, adenine requiring) which complement each other to yield white, non-adenine requiring colonies. Induced mitotic crossing over produces pink and red sectored colonies, which can ben directly counted to yield recombination frequency information.

Zimmerman (423) used this system to verify ultraviolet irradiation and EMS as mutagenic agents which induce crossing over in *Saccharomyces*. In a study (424) examining the activity of DMN (dimethylnitrosamine), DEN (diethylnitrosamine), 1-NA (1-naphthylamine) and 2-NA (2-naphthylamine) in inducing crossing over in yeast, Mayer found that these compounds required conversion for activity. The production of active breakdown products on the Udenfriend hydroxylation medium was shown by the high rate of recombinants in *Saccharomyces*.

A review by Brusick and Mayer (425) presents results of the testing of a series of compounds for gene conversion, forward and reverse mutation in *Saccharomyces*, and a discussion of the advantages and disadvantages of using yeast cells for mutagen screening. (See also "Mutation Induction in Yeast," by R. K. Mortimer and L. R. Manney, *Chemical Mutagens; Principles and Methods for Their Detection*, Vol. 1, A. Hollaender, Ed., Plenum Press, 1971.)

Genetic Damage in Other Fungi:

Other fungi besides *Saccharomyces* have been used to study the toxic effects of chemicals for both growth inhibition and genetic effects. Synkavete (426) (Na_4-2-methyl-1,4-naphthohydroquinone diphosphric acid ester) was assayed for toxicity against *Histoplasma*, *Norcardia*, *Aspergillus*, *Tricophyton*, and *Microsporon* species. The fungi were grown on agar containing the compound. Fungal growth was inhibited at 0.15 to 1.20 mg/ml; the compound was toxic to mice at 0.4 mg/g.

Maneb, a commercial fungicide, was reported (427) to exert a toxic effect on *Colletotrichum* through alteration in amino acid, succinic acid, and phosphorus metabolism and production. The mycelial dry weight of *Aspergillus niger* was used to indicate that dinitrobenzene is toxic to that organism (428) at 1.0 μM. The toxicity was attributed to the depression of citric acid cycle intermediates.

A group of benzimidazole and thiophanate fungicides were assayed for toxicity through their ability to induce genetic segregation in *Aspergillus nidulans* grown on media containing the chemicals (429). This effect, probably caused by nondisjunction events, is detected by the appearance of sectors with white or yellow conidia from a treated heterozygous green strain. Benzimidazole, which is mutagenic to other organisms, was both nontoxic and noneffective in causing genetic segregation in Aspergillus. Five derivatives of benzimidazole, MBC, benomyl, TBZ, DTFB, and TTFB were toxic at low concentrations.

The two carbonate derivatives (MBC and benomyl) increased the frequency of sectoring; the two fluoromethyl derivatives (DTFB and TTFB) did not. Thiophanate and two derivatives were also very active genetically. The authors state that the genetic activity may be the cause of the toxicity, and that the widespread use of these fungicides may introduce a genetic hazard.

Another nondisjunction detection system (430) was used to assay 110 pharmaceutical specialties. A strain of Aspergillus containing recessive genes for resistance to an antimetabolite yields, after crossing over or nondisjunction, conidia which will grow on media containing the compound. The two types of events can be distinguished from the colony color and other physiological properties. Using agar diffusion plate assays, the workers detected four groups of compounds which showed "mutagenic" effects. Quinolines, sulfa drugs, benzodiazepines, and pyrazolidines contained active compound members.

Fungi are amenable to many different mutational tests, covering changes in quality, quantity and arrangement of genetic material, and as such may have more relevance to man than the bacterial test systems.

PROTOZOA

Free-living protozoa have been in use as "test animals" since the early 1900's. In 1913, Woodruff and Underhill initiated the use of the term "biological indicators" to describe this use. More recently, Hutner et al. (431) have urged the use of particle-ingesting protozoa in cytotoxic chemical screens because of their exceptional ability to utilize high-molecular weight and fat-soluble materials. The use of protozoans as test organisms in toxicity studies would appeear to bridge the gap between undifferentiated prokaryotic organisms such as bacteria, and the more complex metazoa. Protozoa possess highly developed and specialized organelles for motility and reproduction. They lend themselves to many kinds of biological research since protozoan cell structure, metabolic functions, and nutritional requirements are similar to that of mammalian cells in many instances. Some correlations have been made in terms of response to agents, and protozoa have been effectively utilized in testing the toxicity of various substances.

Tetrahymena pyriformis has been the object of most of these studies in toxicity evaluations. Observations have been made of this organism, and others, in terms of growth and survival, metabolism, gross and ultra morphology, and ciliastic action and movement. The effects of chemicals on the growth of protozoa is measured in terms of change in culture turbidity, production of inhibition zones, and change in direct cell counts. Death of the cells is ascertained by dilution subculturing, staining characteristics and cessation of ciliary beat. Studies of the effects of chemicals on growth and survival are reviewed first.

In a study (432) on hypocholesteremic agents, protozoa, both sterol synthesizing and nonsynthesizing, were used to compare inhibition of growth with the inhibition of cholesterol synthesis as seen in mammals. Ochromonas, Euglena, and Tetrahymena were inhibited by triparonal and benzmalecene, and the authors feel the organisms may be useful in screening for such compounds.

Growth inhibition of Ochromonas sp. (433) was used to study the toxicity of thalidomide and its breakdown products. It was concluded that the mechanism of toxicity of these compounds may be through interference of cellular oxidation. The authors state that "a protozoan test system is useful for studying the potential 'side actions' of drugs in higher animals . . . with some drugs they mimic man and other animals in their response." However, this interpretation cannot be applied indiscriminately. It was found, for example, that cycloheximide has no toxic effect on Amoeba, even at a concentration of 3 mg/ml (434). This chemical has been found to affect 80S ribosomes. Amoeba have 70S ribosomes.

Tetrahymena pyriformis was used to assess the toxicity of mycotoxins. A 60% inhibition of growth was produced by Rubratoxin B at 50 µg/ml. Aflatoxin B and Ochratoxin (435) were not as effective in inhibiting growth as was Rubratoxin and it was found that these mycotoxins had little effect on cell respiration.

A group of purine antagonists was tested for the inhibition of growth of Tetrahymena (436). Those compounds which were inhibitory were found to affect acetate metabolism and sterol synthesis. Some of the analogs (protopine, phenylbutyric acid) cause "suicide" of the cells. This phenomenon does not fit the pattern of the other inhibitors. Another antimetagolite, 5,6-dimethylbenzimidazole, caused growth inhibition in Endamoeba histolytica (437), when the cells were treated with the compound in the media.

In a series of papers on the use of microorganisms (endpoint = growth inhibition) in antitumor agent screens (410, 412), it was reported that, although mammalian cell cultures appear to be superior to microbiological systems (fewer false positives), Tetrahymena pyriformis picked up 82% of the positive compounds. Foley et al. felt that the compounds missed by the protozoan might require physiological alteration by the host for activity.

Johnston et al. (438) have published data on the use of several species of protozoa in a chemotherapeutic agent screen. Euglena, Ochromonas, and Tetrahymena were compared to mammalian cell cultures. The authors consider these to be morphologically homologous. Autobiographic and turbidimetric measurements were used to measure toxic effects. In agar systems, the protozoa detected more known positives than did the mammalian cells. In an assay conducted by Price et al. (439), however, HeLa cells were more efficient and more selective in pinpointing antitumor compounds than were the protozoans. A close relationship was found although, in degree of inhibition, between an agent's HeLa cytotoxicity and its antiprotozoan activity.

Jacob (440) has stated that the growth of Tetrahymena in pure culture may be compared to the growth of somatic tissue cells. She tested a group of aminoazobenzene dyes and metabolic inhibitors against T. pyriformis for growth inhibition.

Another use for Tetrahymena as "test animals" has been in connection with antimalarials. Clancey describes the lethal and growth inhibitory properties of five different compounds (441).

Some investigators have focused on the structural changes effected by toxicants. In these studies, the treated cells are observed directly for toxic effects. In addition to changes in cell and organelle structure, observations on ciliary beat and motility of the cells have been used to assess chemical toxicity.

When 4-nitroquinoline 1-oxide and 4-aminoquinoline 1-oxide were tested against Tetrahymena, it was found that the effect of the active compound (4NQO) was on protein synthesis in the cytoplasm, resulting from the loss of ribosomes (442). In an earlier study by the same group, it was found that of a group of 4NQO derivatives, the proven carcinogenic substances produced anomalies in cell division, while those which were noncarcinogenic did not.

Ethidium bromide, an antitryposomal compound, was assessed for its effect on growth and production of cell changes in protozoa. In Tetrahymena (443), the drug produced changes in mitochondrial morphology and appeared to arrest cell division via reversible effect on DNA synthesis. Both DNA and RNA replication were inhibited after treatment in Trypanosoma mega and Crithidia luciliae, resulting in growth inhibition (444).

In a study with benzopyrene and other polynuclear aromatic hydrocarbons in combination with ultraviolet irradiation (445), it was noted that a correlation exists between the extent of photodynamic toxicity evidenced by morphological changes and loss of motility in protozoa and carcinogenicity in mammals. The effects of metallic ions (Cu, Zn, and Ph) were evaluated with Paramecium (446). The organisms were so sensitive to a plasma membrane injury caused by these ions that the author recommends their use as indicators of low-level toxicity due to such ions in solutions.

Metrazol, a pharmaceutical, was used to treat Amoeba proteus cells which were suspended in a chamber slide in media containing the drug (447). Changes in locomotion and in membrane potential were observed, and it was concluded that these changes were due to disruptions in potassium transport.

The effect of some carcinogens, including aminofluorenes and naphthylamines, on the growth, respiration, and enzyme activity in Tetrahymena was studied (448). The data obtained were ambiguous; different results were obtained in synthetic than in natural media. Carcinogenic mycotoxins were used to treat Tetrahymena pyriformis (435). The three compounds tested were weakly toxic to the cells, and only a marginal effect on their respiration was noted.

The widest use of protozoans in toxicity testing has been in assaying cigarette smoke. These studies have employed observations on ciliary beat of the cells and morphological changes. Wang has suggested that ciliated protozoa may be compared with human respiratory epithelium, in their response to the effects of smoke. In this type of study, the cells are exposed to residue in broth media or to gaseous phase component in perfusion chambers. In tests using nontobacco cigarettes, Kennedy and Elliott (449) reported that the collected residues produced a reduction in motility and ciliary beat, reduction in O_2 consumption and structural degradation of the mitochondria. In an earlier study (450), these effects were shown when the cells were exposed to tobacco smoke. The cilia toxic effects of tobacco smoke have been demonstrated in cells from a number of different phyla. In a series of studies by Weiss (451, 452), similar work was conducted with Paramecium aurelium. Observations were made of motility and cellular disintegration. It was found that the gas phase contains the major portion of the toxic substances.

It is evident that the use of protozoa in toxicity screens should be increased, and efforts made to utilize their unique properties. Some valuable information has already been gained and new assays can be built upon those reported here.

PLANTS

The use of plant material to assay chemicals for toxicity with regard to effects in mammalian systems is not well established. Differences in uptake mechanisms, permeability, repair systems, structure, and the unique CO_2 fixation of plants preclude direct correlation. Even so, with the continuing development of undifferentiated plant cell cultures utilizing defined synthetic media, it is possible to pinpoint the toxic effects of chemicals on metabolic pathways common to plants and animals.

Growth Inhibition:

Plant tissues have been used to study the toxic and physiological effects of herbicides. The in vitro plant systems which can be employed include whole plants, plant segments, isolated leaf cells, and cell cultures. Whole plants may be grown in a solution or emulsion of the chemical and the rate of growth through fresh weight measurements and/or survival studied. Rice plants were tested in this manner against propanil in combination with parathion or paraoxon (453). It was found that parathion and paraoxon block the hydrolysis of propanil by the plants. Similar protective mechanisms occur in other systems.

To study the effect of compounds on the growth and survival of plant cells in tissue culture, small plugs are cut from the cotyledons and placed on agar nutrient media or in a liquid nutrient media. In two studies (454, 455), using Glycine max (soybean) and Populus deltoides (cottonwood) in tissue culture, picloram, 2,4,5-T and dicamba were assayed for their comparative toxic effects. Growth of plant cells in tissue culture on synthetic media makes it possible to isolate specific enzymes, metabolic byproducts, total protein, etc., for study of the physiological effects of chemicals (see H.E. Street, "Plant Cell Cultures, Their Potential for Metabolic Studies," in Biosynthesis and Its Control in Plants, B. V. Milborrow, ed.).

Tissue levels of nitrate and nitrate reductase in the detached leaves of Hordeum vulgare L, after exposure to DCMU (1,1-dimethyl-3-(3,4-dichlorophenyl)-urea) and the triazines, Simazine and Atrazine, were studied by Aslam and Huffaker (456). These chemicals are known inhibitors of photosynthesis (suppressing the fixation of CO_2) and the effect on nitrate reduction is a result of this inhibition.

Effects On Chromosomal Material:

In contrast to the preceding studies which seem to have little relationship with nonphotosynthetic systems, the use of plant cells to study the effects of chemicals on chromosomal material seems more pertinent.

B. K. Vig (457, 458) has developed a method for assessing the occurrence of chromosomal disturbances, including somatic crossing over, in soybean (Glycine max). The seeds are soaked in a solution of the compound under study, allowed to germinate, and the compound and first simple leaves examined for "spots." Ethylmethane sulfonate, daunomycin, and a group of DNA synthesis inhibitors (caffeine, actinomycin, puromycin, cytosine arabinoside, FDU) were tested for their effects in this system. Through comparison of their effects, Vig concluded that a specific event in DNA repair is responsible for the complementary exchanges picked up in the soybean system.

The excellent visualization of chromosomal material in plants make them ideal for mutagenesis studies, and some correlations with human cells can be made. B. S. Kilman (459) reviews the use of root tips to study the effects of chemicals on chromosomes and asserts that they should be regarded as the ideal plant tissue for studying such effects due to ease of handling, presence of a large number of dividing cells and potentiality for direct exposure. A good correlation between the chromosome breaking activity of chemicals in plant and in animal cells appears to exist, although the type of effect and concentration necessary to produce that effect may be very different. Chromosomal damages in studies of this type are determined through microscopic examination of stained chromosomal preparations of colchicine-treated or untreated plant cells in metaphase or anaphase.

In a study by Sturelid (460) the chromosome breakage caused by TEPA (an aziridine derivative) and its analogues in Vicia faba and Chinese hamster cells was compared. The plant cell material for chromosomal analyses was obtained from the lateral root tips of 9 day old seedlings which had been soaked in a solution of the compound for two hours. The hamster cells were grown and treated as monolayer cultures. They found that the mammalian cells were 23 times more sensitive than the plant cells to the effect of TEPA, although this difference could have been due to the differences in treatment temperature. Although the types of aberrations seen in the two preperations differed and was influenced by temperature, both arose from lesions produced in the chromosomes followed by a period of DNA synthesis with conversion to visible chromatid aberrations. These alkylating agents induce mutations and/or chromosomal aberrations in *Drosophila*, *Neurospora*, *Allium*, and in mouse and human cultured cells.

In another study by Sturelid (461) on the differential reaction of *Allium* root tips and Chinese hamster cells to treatment with caffeine, 3-ethoxycaffeine (EOC) and 6-methylcoumarin (6-MC), it was found that, again, temperature influenced the type and degree of damage. At 17°C both plant and animal cells contained subchromatid and chromatid exchanges, although the animal cells suffered a greater degree of this damage. With 37°C treatment, chromatid fragmentation was produced in the animal cells. The effects of caffeine as a potentiator for damage by other agents was studied in *Vicia faba* root tips (284). It was found that caffeine inhibits the DNA repair gap filling process, and therefore increases chromosomal aberration frequencies.

Phleomycin, a compound which blocks replication of HeLa cells, was tested for its effect in cultured lily cells (462). This compound was found to affect *Allium* similarly, blocking the progression of the chromosomes from one organizational form to another. A study (463) using onion root tip cells (*Allium cepa*), treated with reserpine phosphate, showed that the effect of this alkaloid was mitotic arrest in late prophase. The compound was toxic at 4 mg% to the germinating bulbs.

Algae Cells:

In addition to the use of plant tissue for toxicity testing, some work has been done utilizing eucaryotic algae to study effects on cell growth. *Chlorella vulgaris* has been used to assay the activity of soil herbicides (464). .01 ppm of diuron and 0.5 ppm of monuron caused 50% growth inhibition. *Chlorella pyrenoidosa* was treated with a group of quinone derivatives in broth culture (465). Observations were made of effects on cell counts, chlorophyl concentration, oxygen evolution, and viability. A group of unicellular algae were assayed for the effects of polychlorinated biphenyls (466). The growth of diatoms was inhibited more by PCB's than by DDT. Two fresh water and one salt water algae were found to be resistant to PCB's and DDT.

In attempting to use plant tissue for study of toxicity of chemicals and to apply the results to mammalian tissues, the inherent biochemical and structural differences must be considered. Temperature, pH, oxygen, tension and age of the material are also factors which greatly influence the effects produced. Obviously, more correlative data must be accumulated before the feasibility of using these systems is decided.

DISCUSSION

The use of nonmammalian cell systems for toxicity testing is now well established. The use of protozoa for studies of toxicity of smoke elements, of bacteria in cancer chemotherapeutic screens, and of *Saccharomyces* and *Salmonella* in mutagen screens is well documented and standardized. Many investigators feel that the data obtained from use of these microorganisms can be applied to what occurs in mammals.

The advantages of using single cell toxicity test systems are many. Of most importance is time. Toxic chemical assessment in whole animals is a lengthy procedure, whereas the cellular systems yield results in 24-48 hours. Large numbers of individuals can be screened concurrently, and several different toxicity indicators observed in a single experiment. Single cell systems can be tailored to give a higher probability of toxicity detection; for example, nutritionally deficient strains are used to examine specific antimetabolities. The cost of running assays is greatly reduced from that in whole animals. Most of these tests can be performed by laboratory technicians with little prior training.

The main obstacle to extrapolation of nonmammalian cell system screen data to mammals lies in the lack of knowledge of what actually occurs in the whole animal. Uptake, transportation, detoxification, metabolic alteration, potentiation, and elimination of the compound are processes which occur only in the whole animal and can profoundly alter the effect of a chemical on the ultimate target cell or system. Provisions must be made for these events when using simplified systems; i.e., the use of metabolic activators and of reactive compound derivatives in the mutagen screens.

Further work on protozoan test systems would be advantageous. These organisms are more closely related to mammalian systems than bacteria, have complex structural and metabolic properties, and yet are easy to manipulate. The development of bacterial general toxicity screens would be useful since their metabolism has been so well studied. Further refinement of the mutagen screen system is in progress.

It is obvious that all of the enormous number of new chemicals entering the human environment, in addition to the untested ones already with us, cannot be tested in mammals. The resources for such a task, in time, in personnel, or in money, simply do not exist. The work presented here shows that usable information about chemicals can be obtained from bacterial, fungal, plant cell, and protozoan test systems.